

CA99/1147

PCT/CA/ 99 / 01147
1 MAR 2000 (01.03.00)

PA 209398

REC'D 14 MAR 2000

WPO

PCT

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

February 15, 2000

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM
THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK
OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT
APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A
FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/110,438

FILING DATE: December 01, 1998

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)



By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

P. SWAIN
Certifying Officer

APPROV

Please type a plus sign (+) inside this box → ☐

PTO/SB/16 (12-97)
Approved for use through 1/31/98. OMB 0651-0037
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)					
Given Name (first and middle (if any))		Family Name or Surname		Residence (City and either State or Foreign Country)	
Andrew D		Murdin		146 Rhodes Circle Newmarket Ontario, Canada L3X 1V2	
Raymond P		Oomen		RR No 1 Schomberg, Ontario, Canada	
LOG 110					
<input type="checkbox"/> Additional Inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (280 characters max)					
Chlamydia Antigens and Corresponding DNA Fragments and Uses Thereof					
CORRESPONDENCE ADDRESS					
Direct all correspondence to:					
<input type="checkbox"/> Customer Number				Place Customer Number Bar Code Label here	
OR Type Customer Number here					
<input checked="" type="checkbox"/> Firm or Individual Name		Gavin R. Zealey			
Address		1755 Steeles Ave West			
Address					
City		Toronto		State	Ontario
Country		Canada		ZIP	M2R 3T4
		Telephone	416-667-2854	Fax	416-667-2860
ENCLOSED APPLICATION PARTS (check all that apply)					
<input checked="" type="checkbox"/> Specification Number of Pages		31		<input type="checkbox"/> Small Entity Statement	
<input checked="" type="checkbox"/> Drawing(s) Number of Sheets		14		<input type="checkbox"/> Other (specify) _____	
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)					
<input type="checkbox"/> A check or money order is enclosed to cover the filing fees				FILING FEE AMOUNT (\$)	
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 50-0244				150.00	
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input checked="" type="checkbox"/> No.					
<input type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: _____					

Respectfully submitted,

SIGNATURE _____

TYPED or PRINTED NAME Gavin R. Zealey

TELEPHONE 416-667-2854

Date 10/27/98

REGISTRATION NO. 39,475
(if appropriate)
Docket Number: RY-50

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, DC 20231.

TITLE OF INVENTION

CHLAMYDIA ANTIGENS AND CORRESPONDING DNA FRAGMENTS AND USES
THEREOF

FIELD OF INVENTION

5 The present invention relates to *Chlamydia* antigens and corresponding DNA molecules, which can be used in methods to prevent and treat *Chlamydia* infection in mammals, such as humans.

BACKGROUND OF THE INVENTION

10 Chlamydiae are prokaryotes. They exhibit morphologic and structural similarities to gram-negative bacteria including a trilaminar outer membrane, which contains lipopolysaccharide and several membrane proteins that are structurally and functionally analogous to proteins found in *E coli*. They are obligate intra-cellular parasites with a unique biphasic life cycle consisting of a metabolically inactive but infectious extracellular stage and a replicating but non-infectious intracellular stage. The replicative stage of the life-cycle takes
15 place within a membrane-bound inclusion which sequesters the bacteria away from the cytoplasm of the infected host cell.

20 *C. pneumoniae* is a common human pathogen, originally described as the TWAR strain of *Chlamydia psittaci* but subsequently recognised to be a new species. *C. pneumoniae* is antigenically, genetically and morphologically distinct from other chlamydia species (*C. trachomatis*, *C. pecorum* and *C. psittaci*). It shows 10% or less DNA sequence homology with either of *C. trachomatis* or *C. psittaci* and so far appears to consist of only a single strain, TWAR.

25 *C. pneumoniae* is a common cause of community acquired pneumonia, only less frequent than *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* (Ref 1,2). It can also cause upper respiratory tract symptoms and disease, including bronchitis and sinusitis (Ref 1,3,4,5). The great majority of the adult population (over 60%) has antibodies to *C. pneumoniae* (Ref 5), indicating past infection which was unrecognized or asymptomatic.

30 Of considerable importance is the association of atherosclerosis and *C. pneumoniae* infection. There are several epidemiological studies showing a correlation of previous infections with *C. pneumoniae* and heart attacks, coronary artery and carotid artery disease (Ref 6-10). Moreover, the organisms has been detected in atheromas and fatty streaks of the coronary, carotid, peripheral arteries and aorta (Ref 11-15). Viable *C. pneumoniae* has been recovered

from the coronary and carotid artery (Ref 16,17). Furthermore, it has been shown that *C. pneumoniae* can induce changes of atherosclerosis in a rabbit model (Ref 18). Taken together, these results indicate that it is highly probable that *C. pneumoniae* can cause atherosclerosis in humans, though the epidemiological importance of chlamydial atherosclerosis remains to be demonstrated.

A number of recent studies have also indicated an association between *C. pneumoniae* infection and asthma. Infection has been linked to wheezing, asthmatic bronchitis, adult-onset asthma and acute exacerbations of asthma in adults, and small-scale studies have shown that prolonged antibiotic treatment was effective at greatly reducing the severity of the disease in some individuals (Ref 19-24).

In light of these results a protective vaccine against *C. pneumoniae* infection would be of considerable importance. There is not yet an effective vaccine for any human chlamydial infection. Nevertheless, studies with *C. trachomatis* and *C. psittaci* indicate that this is an attainable goal. For example, mice which have recovered from a lung infection with *C. trachomatis* are protected from infertility induced by a subsequent vaginal challenge (Ref 25). Similarly, sheep immunized with inactivated *C. psittaci* were protected from subsequent chlamydial-induced abortions and stillbirths (Ref 26). Protection from chlamydial infections has been associated with Th1 immune responses, particularly the induction of INF γ - producing CD4+T-cells (Ref 27). The adoptive transfer of CD4+ cell lines or clones to nude or SCID mice conferred protection from challenge or cleared chronic disease (Ref 28,29), and in vivo depletion of CD4+ T cells exacerbated disease post-challenge (Ref 30,31). However, the presence of sufficiently high titres of neutralising antibody at mucosal surfaces can also exert a protective effect (Ref 32).

The extent of antigenic variation within the species *C. pneumoniae* is not well characterised. Serovars of *C. trachomatis* are defined on the basis of antigenic variation in MOMP, but published *C. pneumoniae* MOMP gene sequences show no variation between several diverse isolates of the organism (Ref 33-35). Regions of the protein known to be conserved in other chlamydial MOMPs are conserved in *C. pneumoniae* (Ref 33,34). One study has described a strain of *C. pneumoniae* with a MOMP of greater than usual molecular weight, but the gene for this has not been sequenced (Ref 1). Partial sequences of outer membrane protein 2 from nine diverse isolates were also found to be invariant (Ref 16). The genes for

HSP60 and HSP70 show little variation from other chlamydial species, as would be expected. The gene encoding a 76kDa antigen has been cloned from a single strain of *C. pneumoniae*. It has no significant similarity with other known chlamydial genes (Ref 4).

Many antigens recognised by immune sera to *C. pneumoniae* are conserved across all chlamydiae, but 98kDa, 76 kDa and 54 kDa proteins may be *C. pneumoniae*-specific (Ref 2, 4, 36). Immunoblotting of isolates with sera from patients does show variation of blotting patterns between isolates, indicating that serotypes *C. pneumoniae* may exist (Ref 1,16). However, the results are potentially confounded by the infection status of the patients, since immunoblot profiles of a patient's sera change with time post-infection. An assessment of the number and relative frequency of any serotypes, and the defining antigens, is not yet possible.

C. pneumoniae infection usually presents as an acute respiratory disease (i.e., cough, sore throat, hoarseness, and fever; abnormal chest sounds on auscultation). For most patients, the cough persists for 2 to 6 weeks, and recovery is slow. In approximately 10% of these cases, upper respiratory tract infection is followed by bronchitis or pneumonia. Furthermore, during a *C. pneumoniae* epidemic, subsequent co-infection with pneumococcus has been noted in about half of these pneumonia patients, particularly in the infirm and the elderly. As noted above, there is more and more evidence that *C. pneumoniae* infection is also linked to diseases other than respiratory infections.

The reservoir for the organism is presumably people. In contrast to *C. psittaci* infections, there is no known bird or animal reservoir. Transmission has not been clearly defined. It may result from direct contact with secretions, from fomites, or from airborne spread. There is a long incubation period, which may last for many months. Based on analysis of epidemics, *C. pneumoniae* appears to spread slowly through a population (case-to-case interval averaging 30 days) because infected persons are inefficient transmitters of the organism. Susceptibility to *C. pneumoniae* is universal. Reinfections occur during adulthood, following the primary infection as a child. *C. pneumoniae* appears to be an endemic disease throughout the world, noteworthy for superimposed intervals of increased incidence (epidemics) that persist for 2 to 3 years. *C. trachomatis* infection does not confer cross-immunity to *C. pneumoniae*. Infections are easily treated with oral antibiotics, tetracycline or erythromycin (2 g/d, for at least 10 to 14 d). A recently developed drug, azithromycin, is highly effective as a single-dose therapy against chlamydial infections.

In most instances, *C. pneumoniae* infection is often mild and without complications, and up to 90% of infections are subacute or unrecognized. Among children in industrialized countries, infections have been thought to be rare up to the age of 5 y, although a recent study (E Normann et al, *Chlamydia pneumoniae* in children with acute respiratory tract infections, *Acta Paediatrica*, 1998, Vol 87, Iss 1, pp 23-27) has reported that many children in this age group show PCR evidence of infection despite being seronegative, and estimates a prevalence of 17-19% in 2-4 y olds. In developing countries, the seroprevalence of *C. pneumoniae* antibodies among young children is elevated, and there are suspicions that *C. pneumoniae* may be an important cause of acute lower respiratory tract disease and mortality for infants and children in tropical regions of the world.

From seroprevalence studies and studies of local epidemics, the initial *C. pneumoniae* infection usually happens between the ages of 5 and 20 y. In the USA, for example, there are estimated to be 30,000 cases of childhood pneumonia each year caused by *C. pneumoniae*. Infections may cluster among groups of children or young adults (e.g., school pupils or military conscripts).

C. pneumoniae causes 10 to 25% of community-acquired lower respiratory tract infections (as reported from Sweden, Italy, Finland, and the USA). During an epidemic, *C. pneumoniae* infection may account for 50 to 60% of the cases of pneumonia. During these periods, also, more episodes of mixed infections with *S. pneumoniae* have been reported.

Reinfection during adulthood is common; the clinical presentation tends to be milder. Based on population seroprevalence studies, there tends to be increased exposure with age, which is particularly evident among men. Some investigators have speculated that a persistent, asymptomatic *C. pneumoniae* infection state is common.

In adults of middle age or older, *C. pneumoniae* infection may progress to chronic bronchitis and sinusitis. A study in the USA revealed that the incidence of pneumonia caused by *C. pneumoniae* in persons younger than 60 years is 1 case per 1,000 persons per year; but in the elderly, the disease incidence rose three-fold. *C. pneumoniae* infection rarely leads to hospitalization, except in patients with an underlying illness.

SUMMARY OF THE INVENTION

The present invention provides purified and isolated DNA molecules that encode *Chlamydia* polypeptides designated CPN100635 (SEQ ID No: 1,2), which can be used in

methods to prevent, treat, and diagnose *Chlamydia* infection. The encoded polypeptides include polypeptides having the amino acid sequence shown in SEQ ID No:3 and 4. Those skilled in the art will appreciate that the invention also includes DNA molecules that encode mutants and derivatives of such polypeptides, which result from the addition, deletion, or substitution of non-essential amino acids as described herein. The invention also includes RNA molecules corresponding to the DNA molecules of the invention.

In addition to the DNA and RNA molecules, the invention includes the corresponding polypeptides and monospecific antibodies that specifically bind to such polypeptides.

The present invention has wide application and includes expression cassettes, vectors, and cells transformed or transfected with the polynucleotides of the invention. Accordingly, the present invention provides (i) a method for producing a polypeptide of the invention in a recombinant host system and related expression cassettes, vectors, and transformed or transfected cells; (ii) a live vaccine vector, such as a pox virus, *Salmonella typhimurium*, or *Vibrio cholerae* vector, containing a polynucleotide of the invention, such vaccine vectors being useful for, e.g., preventing and treating *Chlamydia* infection, in combination with a diluent or carrier, and related pharmaceutical compositions and associated therapeutic and/or prophylactic methods; (iii) a therapeutic and/or prophylactic method involving administration of an RNA or DNA molecule of the invention, either in a naked form or formulated with a delivery vehicle, a polypeptide or combination of polypeptides, or a monospecific antibody of the invention, and related pharmaceutical compositions; (iv) a method for diagnosing the presence of *Chlamydia* in a biological sample, which can involve the use of a DNA or RNA molecule, a monospecific antibody, or a polypeptide of the invention; and (v) a method for purifying a polypeptide of the invention by antibody-based affinity chromatography.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be further understood from the following description with reference to the drawings, in which:

Figure 1 shows the nucleotide sequence of the CPN100635 (SEQ ID No: 1 – entire sequence and SEQ ID No: 2 – coding sequence) and the deduced amino acid sequence of the CPN100635 protein from *Chlamydia pneumoniae* (SEQ ID No: 3 - unprocessed and 4 - processed).

Figure 2 shows the restriction enzyme analysis of the gene encoding the *C. pneumoniae* CPN100635 gene.

DETAILED DESCRIPTION OF INVENTION

In the *C. pneumoniae* genome, open reading frames (ORFs) encoding chlamydial polypeptides have been identified. These polypeptides include polypeptides permanently found in the bacterial membrane structure, polypeptides that are present in the external vicinity of the bacterial membrane, include polypeptides permanently found in the inclusion membrane structure, polypeptides that are present in the external vicinity of the inclusion membrane, and polypeptides that are released into the cytoplasm of the infected cell. These polypeptides can be used in vaccination methods for preventing and treating *Chlamydia* infection.

According to a first aspect of the invention, there are provided isolated polynucleotides encoding the precursor and mature forms of *Chlamydia* polypeptides.

An isolated polynucleotide of the invention encodes (i) a polypeptide having an amino acid sequence that is homologous to a *Chlamydia* amino acid, the *Chlamydia* amino acid sequence being selected from the group consisting of:

(a) the amino acid sequences as shown: (SEQ ID No: 3 and 4)

The term "isolated polynucleotide" is defined as a polynucleotide removed from the environment in which it naturally occurs. For example, a naturally-occurring DNA molecule present in the genome of a living bacteria or as part of a gene bank is not isolated, but the same molecule separated from the remaining part of the bacterial genome, as a result of, e.g., a cloning event (amplification), is isolated. Typically, an isolated DNA molecule is free from DNA regions (e.g., coding regions) with which it is immediately contiguous at the 5' or 3' end, in the naturally occurring genome. Such isolated polynucleotides could be part of a vector or a composition and still be isolated in that such a vector or composition is not part of its natural environment.

A polynucleotide of the invention can be in the form of RNA or DNA (e.g., cDNA, genomic DNA, or synthetic DNA), or modifications or combinations thereof. The DNA can be double-stranded or single-stranded, and, if single-stranded, can be the coding strand or the non-coding (anti-sense) strand. The sequence that encodes a polypeptide of the invention as shown in SEQ ID NOs: 1 and 2, can be (a) the coding sequence as shown in SEQ ID NOs:2 (b) a ribonucleotide sequence derived by transcription of (a) ; or (c) a different coding sequence; this

latter, as a result of the redundancy or degeneracy of the genetic code, encodes the same polypeptides as the DNA molecules of which the nucleotide sequences are illustrated in SEQ ID NOs:1 to 2.

5 By "polypeptide" or "protein" is meant any chain of amino acids, regardless of length or post-translational modification (*e.g.*, glycosylation or phosphorylation). Both terms are used interchangeably in the present application.

10 By "homologous amino acid sequence" is meant an amino acid sequence that differs from an amino acid sequence shown in SEQ ID No: 3 or 4, only by one or more conservative amino acid substitutions, or by one or more non-conservative amino acid substitutions, deletions, or additions located at positions at which they do not destroy the specific antigenicity of the polypeptide.

Preferably, such a sequence is at least 75%, more preferably 80%, and most preferably 90% identical to an amino acid sequence shown in SEQ ID No: 3 or 4.

15 Homologous amino acid sequences include sequences that are identical or substantially identical to an amino acid sequence as shown in SEQ ID No:3 or 4. By "amino acid sequence substantially identical" is meant a sequence that is at least 90%, preferably 95%, more preferably 97%, and most preferably 99% identical to an amino acid sequence of reference and that preferably differs from the sequence of reference, if at all, by a majority of conservative amino acid substitutions.

20 Conservative amino acid substitutions typically include substitutions among amino acids of the same class. These classes include, for example, amino acids having uncharged polar side chains, such as asparagine, glutamine, serine, threonine, and tyrosine; amino acids having basic side chains, such as lysine, arginine, and histidine; amino acids having acidic side chains, such as aspartic acid and glutamic acid; and amino acids having nonpolar side chains, such as
25 glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, and cysteine.

Homology is typically measured using sequence analysis software (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Similar amino acid
30 sequences are aligned to obtain the maximum degree of homology (*i.e.*, identity). To this end, it may be necessary to artificially introduce gaps into the sequence. Once the optimal alignment

has been set up, the degree of homology (*i.e.*, identity) is established by recording all of the positions in which the amino acids of both sequences are identical, relative to the total number of positions.

Homologous polynucleotide sequences are defined in a similar way. Preferably, a homologous sequence is one that is at least 45%, more preferably 60%, and most preferably 85% identical to (i) a coding sequence of SEQ ID NOs:1 and 2.

Polypeptides having a sequence homologous to one of the sequences shown in SEQ ID NO: 3 or 4, include naturally-occurring allelic variants, as well as mutants or any other non-naturally occurring variants that are analogous in terms of antigenicity, to a polypeptide having a sequence as shown in SEQ ID NO: 3 or 4.

As is known in the art, an allelic variant is an alternate form of a polypeptide that is characterized as having a substitution, deletion, or addition of one or more amino acids that does not alter the biological function of the polypeptide. By "biological function" is meant the function of the polypeptide in the cells in which it naturally occurs, even if the function is not necessary for the growth or survival of the cells. For example, the biological function of a porin is to allow the entry into cells of compounds present in the extracellular medium. The biological function is distinct from the antigenic function. A polypeptide can have more than one biological function.

Allelic variants are very common in nature. For example, a bacterial species, *e.g.*, *C. pneumoniae*, is usually represented by a variety of strains that differ from each other by minor allelic variations. Indeed, a polypeptide that fulfills the same biological function in different strains can have an amino acid sequence that is not identical in each of the strains. Such an allelic variation may be equally reflected at the polynucleotide level.

Support for the use of allelic variants of polypeptide antigens comes from, *e.g.*, studies of the *Chlamydial* MOMP antigen. The amino acid sequence of the MOMP varies from strain to strain, yet cross-strain antibody binding plus neutralization of infectivity occurs, indicating that the MOMP, when used as an immunogen, is tolerant of amino acid variations.

Polynucleotides, *e.g.*, DNA molecules, encoding allelic variants can easily be retrieved by polymerase chain reaction (PCR) amplification of genomic bacterial DNA extracted by conventional methods. This involves the use of synthetic oligonucleotide primers matching upstream and downstream of the 5' and 3' ends of the encoding domain. Suitable primers can be

designed according to the nucleotide sequence information provided in SEQ ID NOs:1 and 2. Typically, a primer can consist of 10 to 40, preferably 15 to 25 nucleotides. It may be also advantageous to select primers containing C and G nucleotides in a proportion sufficient to ensure efficient hybridization; *e.g.*, an amount of C and G nucleotides of at least 40%, preferably
5 50% of the total nucleotide amount.

Useful homologs that do not naturally occur can be designed using known methods for identifying regions of an antigen that are likely to be tolerant of amino acid sequence changes and/or deletions. For example, sequences of the antigen from different species can be compared to identify conserved sequences.

10 Polypeptide derivatives that are encoded by polynucleotides of the invention include, *e.g.*, fragments, polypeptides having large internal deletions derived from full-length polypeptides, and fusion proteins.

Polypeptide fragments of the invention can be derived from a polypeptide having a sequence homologous to any of the sequences shown in SEQ ID NO: 3 or 4 , to the extent that
15 the fragments retain the substantial antigenicity of the parent polypeptide (specific antigenicity). Polypeptide derivatives can also be constructed by large internal deletions that remove a substantial part of the parent polypeptide, while retaining specific antigenicity. Generally, polypeptide derivatives should be about at least 12 amino acids in length to maintain antigenicity. Advantageously, they can be at least 20 amino acids, preferably at least 50 amino
20 acids, more preferably at least 75 amino acids, and most preferably at least 100 amino acids in length.

Useful polypeptide derivatives, *e.g.*, polypeptide fragments, can be designed using computer-assisted analysis of amino acid sequences in order to identify sites in protein antigens having potential as surface-exposed, antigenic regions (Ref 37).

25 Polypeptide fragments and polypeptides having large internal deletions can be used for revealing epitopes that are otherwise masked in the parent polypeptide and that may be of importance for inducing a protective T cell-dependent immune response. Deletions can also remove immunodominant regions of high variability among strains.

It is an accepted practice in the field of immunology to use fragments and variants of
30 protein immunogens as vaccines, as all that is required to induce an immune response to a protein is a small (*e.g.*, 8 to 10 amino acid) immunogenic region of the protein. This has been

done for a number of vaccines against pathogens other than *Chlamydia*. For example, short synthetic peptides corresponding to surface-exposed antigens of pathogens such as murine mammary tumor virus, peptide containing 11 amino acids; (Ref 38), Semliki Forest virus, peptide containing 16 amino acids (Ref 39), and canine parvovirus, 2 overlapping peptides, each
5 containing 15 amino acids (Ref 40), have been shown to be effective vaccine antigens against their respective pathogens.

Polynucleotides encoding polypeptide fragments and polypeptides having large internal deletions can be constructed using standard methods (Ref 41), for example, by PCR, including inverse PCR, by restriction enzyme treatment of the cloned DNA molecules, or by the
10 method of Kunkel *et al.* (Ref 42) biological material available at Stratagene.

A polypeptide derivative can also be produced as a fusion polypeptide that contains a polypeptide or a polypeptide derivative of the invention fused, *e.g.*, at the N- or C-terminal end, to any other polypeptide (hereinafter referred to as a peptide tail). Such a product can be easily obtained by translation of a genetic fusion, *i.e.*, a hybrid gene. Vectors for expressing fusion
15 polypeptides are commercially available, such as the pMal-c2 or pMal-p2 systems of New England Biolabs, in which the peptide tail is a maltose binding protein, the glutathione-S-transferase system of Pharmacia, or the His-Tag system available from Novagen. These and other expression systems provide convenient means for further purification of polypeptides and derivatives of the invention.

Another particular example of fusion polypeptides included in invention includes a polypeptide or polypeptide derivative of the invention fused to a polypeptide having adjuvant activity, such as, *e.g.*, subunit B of either cholera toxin or *E. coli* heat-labile toxin. Several possibilities are can be used for achieving fusion. First, the polypeptide of the invention can be fused to the N-, or preferably, to the C-terminal end of the polypeptide having adjuvant activity.
20 Second, a polypeptide fragment of the invention can be fused within the amino acid sequence of the polypeptide having adjuvant activity.

As stated above, the polynucleotides of the invention encode *Chlamydia* polypeptides in precursor or mature form. They can also encode hybrid precursors containing heterologous signal peptides, which can mature into polypeptides of the invention. By "heterologous signal
30 peptide" is meant a signal peptide that is not found in the naturally-occurring precursor of a polypeptide of the invention.

A polynucleotide of the invention, having a homologous coding sequence, hybridizes, preferably under stringent conditions, to a polynucleotide having a sequence as shown in SEQ ID NOs:1 to 2. Hybridization procedures are, e.g., described in Ausubel *et al.*, (Ref 41), Silhavy *et al.* (Ref 43); Davis *et al.* (ref 44). Important parameters that can be considered for optimizing hybridization conditions are reflected in a formula that allows calculation of a critical value, the melting temperature above which two complementary DNA strands separate from each other Ref 45). This formula is as follows: $T_m = 81.5 + 0.5 \times (\% \text{ G+C}) + 1.6 \log (\text{positive ion concentration}) - 0.6 \times (\% \text{ formamide})$. Under appropriate stringency conditions, hybridization temperature (T_h) is approximately 20 to 40°C, 20 to 25°C, or, preferably 30 to 40°C below the calculated T_m . Those skilled in the art will understand that optimal temperature and salt conditions can be readily determined empirically in preliminary experiments using conventional procedures.

For example, stringent conditions can be achieved, both for pre-hybridizing and hybridizing incubations, (i) within 4-16 hours at 42°C, in 6 x SSC containing 50% formamide or (ii) within 4-16 hours at 65°C in an aqueous 6 x SSC solution (1 M NaCl, 0.1 M sodium citrate (pH 7.0)).

For polynucleotides containing 30 to 600 nucleotides, the above formula is used and then is corrected by subtracting (600/polynucleotide size in base pairs). Stringency conditions are defined by a T_h that is 5 to 10°C below T_m .

Hybridization conditions with oligonucleotides shorter than 20-30 bases do not exactly follow the rules set forth above. In such cases, the formula for calculating the T_m is as follows: $T_m = 4 \times (\text{G+C}) + 2 \times (\text{A+T})$. For example, an 18 nucleotide fragment of 50% G+C would have an approximate T_m of 54°C.

A polynucleotide molecule of the invention, containing RNA, DNA, or modifications or combinations thereof, can have various applications. For example, a DNA molecule can be used (i) in a process for producing the encoded polypeptide in a recombinant host system, (ii) in the construction of vaccine vectors such as poxviruses, which are further used in methods and compositions for preventing and/or treating *Chlamydia* infection, (iii) as a vaccine agent (as well as an RNA molecule), in a naked form or formulated with a delivery vehicle and, (iv) in the construction of attenuated *Chlamydia* strains that can over-express a polynucleotide of the invention or express it in a non-toxic, mutated form.

According to a second aspect of the invention, there is therefore provided (i) an expression cassette containing a DNA molecule of the invention placed under the control of the elements required for expression, in particular under the control of an appropriate promoter; (ii) an expression vector containing an expression cassette of the invention; (iii) a procaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, as well as (iv) a process for producing a polypeptide or polypeptide derivative encoded by a polynucleotide of the invention, which involves culturing a procaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, under conditions that allow expression of the DNA molecule of the invention and, recovering the encoded polypeptide or polypeptide derivative from the cell culture.

A recombinant expression system can be selected from procaryotic and eucaryotic hosts. Eucaryotic hosts include yeast cells (e.g., *Saccharomyces cerevisiae* or *Pichia pastoris*), mammalian cells (e.g., COS1, NIH3T3, or JEG3 cells), arthropods cells (e.g., *Spodoptera frugiperda* (SF9) cells), and plant cells. Preferably, a procaryotic host such as *E. coli* is used. Bacterial and eucaryotic cells are available from a number of different sources to those skilled in the art, e.g., the American Type Culture Collection (ATCC; Rockville, Maryland).

The choice of the expression system depends on the features desired for the expressed polypeptide. For example, it may be useful to produce a polypeptide of the invention in a particular lipidated form or any other form.

The choice of the expression cassette will depend on the host system selected as well as the features desired for the expressed polypeptide. Typically, an expression cassette includes a promoter that is functional in the selected host system and can be constitutive or inducible; a ribosome binding site; a start codon (ATG) if necessary, a region encoding a signal peptide, e.g., a lipidation signal peptide; a DNA molecule of the invention; a stop codon; and optionally a 3' terminal region (translation and/or transcription terminator). The signal peptide encoding region is adjacent to the polynucleotide of the invention and placed in proper reading frame. The signal peptide-encoding region can be homologous or heterologous to the DNA molecule encoding the mature polypeptide and can be specific to the secretion apparatus of the host used for expression. The open reading frame constituted by the DNA molecule of the invention, solely or together with the signal peptide, is placed under the control of the promoter so that transcription and translation occur in the host system. Promoters, signal peptide encoding regions are widely

known and available to those skilled in the art and includes, for example, the promoter of *Salmonella typhimurium* (and derivatives) that is inducible by arabinose (promoter araB) and is functional in Gram-negative bacteria such as *E. coli* (as described in U.S. Patent No. 5,028,530 and in Cagnon *et al.*, (Ref 46); the promoter of the gene of bacteriophage T7 encoding RNA polymerase, that is functional in a number of *E. coli* strains expressing T7 polymerase (described in U.S. Patent No. 4,952,496); OspA lipidation signal peptide ; and RlpB lipidation signal peptide (Ref 47).

The expression cassette is typically part of an expression vector, which is selected for its ability to replicate in the chosen expression system. Expression vectors (*e.g.*, plasmids or viral vectors) can be chosen from those described in Pouwels *et al.* (Cloning Vectors: A Laboratory Manual 1985, Supp. 1987). They can be purchased from various commercial sources.

Methods for transforming/transfecting host cells with expression vectors will depend on the host system selected as described in Ausubel *et al.*, (Ref 41).

Upon expression, a recombinant polypeptide of the invention (or a polypeptide derivative) is produced and remains in the intracellular compartment, is secreted/excreted in the extracellular medium or in the periplasmic space, or is embedded in the cellular membrane. The polypeptide can then be recovered in a substantially purified form from the cell extract or from the supernatant after centrifugation of the recombinant cell culture. Typically, the recombinant polypeptide can be purified by antibody-based affinity purification or by any other method that can be readily adapted by a person skilled in the art, such as by genetic fusion to a small affinity binding domain. Antibody-based affinity purification methods are also available for purifying a polypeptide of the invention extracted from a *Chlamydia* strain. Antibodies useful for purifying by immunoaffinity the polypeptides of the invention can be obtained as described below.

A polynucleotide of the invention can also be useful in the vaccine field, *e.g.*, for achieving DNA vaccination. There are two major possibilities, either using a viral or bacterial host as gene delivery vehicle (live vaccine vector) or administering the gene in a free form, *e.g.*, inserted into a plasmid. Therapeutic or prophylactic efficacy of a polynucleotide of the invention can be evaluated as described below.

Accordingly, in a third aspect of the invention, there is provided (i) a vaccine vector such as a poxvirus, containing a DNA molecule of the invention, placed under the control of

elements required for expression; (ii) a composition of matter containing a vaccine vector of the invention, together with a diluent or carrier; particularly, (iii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a vaccine vector of the invention; (iv) a method for inducing an immune response against *Chlamydia* in a mammal (*e.g.*, a human; alternatively, the method can be used in veterinary applications for treating or preventing *Chlamydia* infection of animals, *e.g.*, cats or birds), which involves administering to the mammal an immunogenically effective amount of a vaccine vector of the invention to elicit an immune response, *e.g.*, a protective or therapeutic immune response to *Chlamydia*; and particularly, (v) a method for preventing and/or treating a *Chlamydia* (*e.g.*, *C. trachomatis*, *C. psittaci*, *C. pneumonia*, *C. pecorum*) infection, which involves administering a prophylactic or therapeutic amount of a vaccine vector of the invention to an individual in need. Additionally, the third aspect of the invention encompasses the use of a vaccine vector of the invention in the preparation of a medicament for preventing and/or treating *Chlamydia* infection.

A vaccine vector of the invention can express one or several polypeptides or derivatives of the invention, as well as at least one additional *Chlamydia* antigen, fragment, homolog, mutant, or derivative thereof. In addition, it can express a cytokine, such as interleukin-2 (IL-2) or interleukin-12 (IL-12), that enhances the immune response (adjuvant effect). Thus, a vaccine vector can include an additional DNA sequence encoding, *e.g.*, a chlamydial antigen, or a cytokine, placed under the control of elements required for expression in a mammalian cell.

Alternatively, a composition of the invention can include several vaccine vectors, each of them being capable of expressing a polypeptide or derivative of the invention. A composition can also contain a vaccine vector capable of expressing an additional *Chlamydia* antigen, or a subunit, fragment, homolog, mutant, or derivative thereof; or a cytokine such as IL-2 or IL-12.

In vaccination methods for treating or preventing infection in a mammal, a vaccine vector of the invention can be administered by any conventional route in use in the vaccine field, particularly, to a mucosal (*e.g.*, ocular, intranasal, oral, gastric, pulmonary, intestinal, rectal, vaginal, or urinary tract) surface or *via* the parenteral (*e.g.*, subcutaneous, intradermal, intramuscular, intravenous, or intraperitoneal) route. Preferred routes depend upon the choice of the vaccine vector. The administration can be achieved in a single dose or repeated at intervals.

The appropriate dosage depends on various parameters understood by skilled artisans such as the vaccine vector itself, the route of administration or the condition of the mammal to be vaccinated (weight, age and the like).

Live vaccine vectors available in the art include viral vectors such as adenoviruses
5 and poxviruses as well as bacterial vectors, *e.g.*, *Shigella*, *Salmonella*, *Vibrio cholerae*,
Lactobacillus, Bacille bilié de Calmette-Guérin (BCG), and *Streptococcus*.

An example of an adenovirus vector, as well as a method for constructing an
adenovirus vector capable of expressing a DNA molecule of the invention, are described in
U.S. Patent No. 4,920,209. Poxvirus vectors that can be used include, *e.g.*, vaccinia and canary
10 pox virus, described in U.S. Patent No. 4,722,848 and U.S. Patent No. 5,364,773, respectively
(also see, *e.g.*, Tartaglia *et al.*, Virology (1992) 188:217) for a description of a vaccinia virus
vector; and Taylor *et al.*, Vaccine (1995) 13:539 for a reference of a canary pox). Poxvirus
vectors capable of expressing a polynucleotide of the invention can be obtained by homologous
recombination as described in Kieny *et al.*, Nature (1984) 312:163 so that the polynucleotide of
15 the invention is inserted in the viral genome under appropriate conditions for expression in
mammalian cells. Generally, the dose of vaccine viral vector, for therapeutic or prophylactic use,
can be of from about 1×10^4 to about 1×10^{11} , advantageously from about 1×10^7 to about 1×10^{10} ,
preferably of from about 1×10^7 to about 1×10^9 plaque-forming units per kilogram. Preferably,
viral vectors are administered parenterally; for example, in 3 doses, 4 weeks apart. Those skilled
20 in the art recognize that it is preferable to avoid adding a chemical adjuvant to a composition
containing a viral vector of the invention and thereby minimizing the immune response to the
viral vector itself.

Non-toxicogenic *Vibrio cholerae* mutant strains that are useful as a live oral vaccine
are described in Mekalanos *et al.*, Nature (1983) 306:551 and U.S. Patent No. 4,882,278 (strain
25 in which a substantial amount of the coding sequence of each of the two *ctxA* alleles has been
deleted so that no functional *cholerae* toxin is produced); WO 92/11354 (strain in which the *irgA*
locus is inactivated by mutation; this mutation can be combined in a single strain with *ctxA*
mutations); and WO 94/1533 (deletion mutant lacking functional *ctxA* and *attRS1* DNA
sequences). These strains can be genetically engineered to express heterologous antigens, as
30 described in WO 94/19482. An effective vaccine dose of a *Vibrio cholerae* strain capable of
expressing a polypeptide or polypeptide derivative encoded by a DNA molecule of the invention

can contain, e.g., about 1×10^5 to about 1×10^9 , preferably about 1×10^6 to about 1×10^8 viable bacteria in an appropriate volume for the selected route of administration. Preferred routes of administration include all mucosal routes; most preferably, these vectors are administered intranasally or orally.

5 Attenuated *Salmonella typhimurium* strains, genetically engineered for recombinant expression of heterologous antigens or not, and their use as oral vaccines are described in Nakayama *et al.* (Bio/Technology (1988) 6:693) and WO 92/11361. Preferred routes of administration include all mucosal routes; most preferably, these vectors are administered intranasally or orally.

10 Others bacterial strains useful as vaccine vectors are described in High *et al.*, EMBO (1992) 11:1991 and Sizemore *et al.*, Science (1995) 270:299 (*Shigella flexneri*); Medaglini *et al.*, Proc. Natl. Acad. Sci. USA (1995) 92:6868 (*Streptococcus gordonii*); and Flynn J.L., Cell. Mol. Biol. (1994) 40 (suppl. I):31, WO 88/6626, WO 90/0594, WO 91/13157, WO 92/1796, and WO 92/21376 (Bacille Calmette Guerin).

15 In bacterial vectors, polynucleotide of the invention can be inserted into the bacterial genome or can remain in a free state, carried on a plasmid.

 An adjuvant can also be added to a composition containing a vaccine bacterial vector. A number of adjuvants are known to those skilled in the art. Preferred adjuvants can be selected from the list provided below.

20 According to a fourth aspect of the invention, there is also provided (i) a composition of matter containing a polynucleotide of the invention, together with a diluent or carrier; (ii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a polynucleotide of the invention; (iii) a method for inducing an immune response against *Chlamydia*, in a mammal, by administering to the mammal, an immunogenically effective
25 amount of a polynucleotide of the invention to elicit an immune response, e.g., a protective immune response to *Chlamydia*; and particularly, (iv) a method for preventing and/or treating a *Chlamydia* (e.g., *C. trachomatis*, *C. psittaci*, *C. pneumoniae*, or *C. pecorum*) infection, by administering a prophylactic or therapeutic amount of a polynucleotide of the invention to an individual in need. Additionally, the fourth aspect of the invention encompasses the use of a
30 polynucleotide of the invention in the preparation of a medicament for preventing and/or treating *Chlamydia* infection. The fourth aspect of the invention preferably includes the use of a DNA

molecule placed under conditions for expression in a mammalian cell, *e.g.*, in a plasmid that is unable to replicate in mammalian cells and to substantially integrate in a mammalian genome.

Polynucleotides (DNA or RNA) of the invention can also be administered as such to a mammal for vaccine, *e.g.*, therapeutic or prophylactic, purpose. When a DNA molecule of the invention is used, it can be in the form of a plasmid that is unable to replicate in a mammalian cell and unable to integrate in the mammalian genome. Typically, a DNA molecule is placed under the control of a promoter suitable for expression in a mammalian cell. The promoter can function ubiquitously or tissue-specifically. Examples of non-tissue specific promoters include the early Cytomegalovirus (CMV) promoter (described in U.S. Patent No. 4,168,062) and the Rous Sarcoma Virus promoter (described in Norton & Coffin, *Molec. Cell Biol.* (1985) 5:281). The desmin promoter (Li *et al.*, *Gene* (1989) 78:243, Li & Paulin, *J. Biol. Chem.* (1991) 266:6562 and Li & Paulin, *J. Biol. Chem.* (1993) 268:10403) is tissue-specific and drives expression in muscle cells. More generally, useful vectors are described, *i.a.*, WO 94/21797 and Hartikka *et al.*, *Human Gene Therapy* (1996) 7:1205.

For DNA/RNA vaccination, the polynucleotide of the invention can encode a precursor or a mature form. When it encodes a precursor form, the precursor form can be homologous or heterologous. In the latter case, a eucaryotic leader sequence can be used, such as the leader sequence of the tissue-type plasminogen factor (tPA).

A composition of the invention can contain one or several polynucleotides of the invention. It can also contain at least one additional polynucleotide encoding another *Chlamydia* antigen such as urease subunit A, B, or both; or a fragment, derivative, mutant, or analog thereof. A polynucleotide encoding a cytokine, such as interleukin-2 (IL-2) or interleukin-12 (IL-12), can also be added to the composition so that the immune response is enhanced. These additional polynucleotides are placed under appropriate control for expression. Advantageously, DNA molecules of the invention and/or additional DNA molecules to be included in the same composition, can be carried in the same plasmid.

Standard techniques of molecular biology for preparing and purifying polynucleotides can be used in the preparation of polynucleotide therapeutics of the invention. For use as a vaccine, a polynucleotide of the invention can be formulated according to various methods.

First, a polynucleotide can be used in a naked form, free of any delivery vehicles, such as anionic liposomes, cationic lipids, microparticles, *e.g.*, gold microparticles, precipitating

agents, *e.g.*, calcium phosphate, or any other transfection-facilitating agent. In this case, the polynucleotide can be simply diluted in a physiologically acceptable solution, such as sterile saline or sterile buffered saline, with or without a carrier. When present, the carrier preferably is isotonic, hypotonic, or weakly hypertonic, and has a relatively low ionic strength, such as
 5 provided by a sucrose solution, *e.g.*, a solution containing 20% sucrose.

Alternatively, a polynucleotide can be associated with agents that assist in cellular uptake. It can be, *i.a.*, (i) complemented with a chemical agent that modifies the cellular permeability, such as bupivacaine (see, *e.g.*, WO 94/16737), (ii) encapsulated into liposomes, or (iii) associated with cationic lipids or silica, gold, or tungsten microparticles.

10 Anionic and neutral liposomes are well-known in the art (see, *e.g.*, Liposomes: A Practical Approach, RPC New Ed, IRL press (1990), for a detailed description of methods for making liposomes) and are useful for delivering a large range of products, including polynucleotides.

Cationic lipids are also known in the art and are commonly used for gene delivery.
 15 Such lipids include Lipofectin™ also known as DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride), DOTAP (1,2-bis(oleyloxy)-3-(trimethylammonio)propane), DDAB (dimethyldioctadecylammonium bromide), DOGS (dioctadecylamidoglycyl spermine) and cholesterol derivatives such as DC-Chol (3 beta-(N-(N',N'-dimethyl aminomethane)-carbamoyl) cholesterol). A description of these cationic lipids can be found in EP 187,702,
 20 WO 90/11092, U.S. Patent No. 5,283,185, WO 91/15501, WO 95/26356, and U.S. Patent No. 5,527,928. Cationic lipids for gene delivery are preferably used in association with a neutral lipid such as DOPE (dioleoyl phosphatidylethanolamine), as, for example, described in WO 90/11092.

Other transfection-facilitating compounds can be added to a formulation containing
 25 cationic liposomes. A number of them are described in, *e.g.*, WO 93/18759, WO 93/19768, WO 94/25608, and WO 95/2397. They include, *i.a.*, spermine derivatives useful for facilitating the transport of DNA through the nuclear membrane (see, for example, WO 93/18759) and membrane-permeabilizing compounds such as GALA, Gramicidine S, and cationic bile salts (see, for example, WO 93/19768).

30 Gold or tungsten microparticles can also be used for gene delivery, as described in WO 91/359, WO 93/17706, and Tang *et al.* (Nature (1992) 356:152). In this case, the

microparticle-coated polynucleotides can be injected *via* intradermal or intraepidermal routes using a needleless injection device ("gene gun"), such as those described in U.S. Patent No. 4,945,050, U.S. Patent No. 5,015,580, and WO 94/24263.

The amount of DNA to be used in a vaccine recipient depends, *e.g.*, on the strength of the promoter used in the DNA construct, the immunogenicity of the expressed gene product, the condition of the mammal intended for administration (*e.g.*, the weight, age, and general health of the mammal), the mode of administration, and the type of formulation. In general, a therapeutically or prophylactically effective dose from about 1 μ g to about 1 mg, preferably, from about 10 μ g to about 800 μ g and, more preferably, from about 25 μ g to about 250 μ g, can be administered to human adults. The administration can be achieved in a single dose or repeated at intervals.

The route of administration can be any conventional route used in the vaccine field. As general guidance, a polynucleotide of the invention can be administered *via* a mucosal surface, *e.g.*, an ocular, intranasal, pulmonary, oral, intestinal, rectal, vaginal, and urinary tract surface; or *via* a parenteral route, *e.g.*, by an intravenous, subcutaneous, intraperitoneal, intradermal, intraepidermal, or intramuscular route. The choice of the administration route will depend on, *e.g.*, the formulation that is selected. A polynucleotide formulated in association with bupivacaine is advantageously administered into muscles. When a neutral or anionic liposome or a cationic lipid, such as DOTMA or DC-Chol, is used, the formulation can be advantageously injected *via* intravenous, intranasal (aerosolization), intramuscular, intradermal, and subcutaneous routes. A polynucleotide in a naked form can advantageously be administered *via* the intramuscular, intradermal, or sub-cutaneous routes.

Although not absolutely required, such a composition can also contain an adjuvant. If so, a systemic adjuvant that does not require concomitant administration in order to exhibit an adjuvant effect is preferable such as, *e.g.*, QS21, which is described in U.S. Patent No. 5,057,546.

The sequence information provided in the present application enables the design of specific nucleotide probes and primers that can be useful in diagnosis. Accordingly, in a fifth aspect of the invention, there is provided a nucleotide probe or primer having a sequence found in or derived by degeneracy of the genetic code from a sequence shown in SEQ ID NO:1 to 2.

The term "probe" as used in the present application refers to DNA (preferably single stranded) or RNA molecules (or modifications or combinations thereof) that hybridize under the

stringent conditions, as defined above, to nucleic acid molecules having sequences homologous to those shown in SEQ ID NOs:1 and 2, or to a complementary or anti-sense sequence. Generally, probes are significantly shorter than full-length sequences shown in SEQ ID NOs:1 and 2; for example, they can contain from about 5 to about 100, preferably from about 10 to about 80 nucleotides. In particular, probes have sequences that are at least 75%, preferably at least 85%, more preferably 95% homologous to a portion of a sequence as shown in SEQ ID NOs:1 and 2 or that are complementary to such sequences. Probes can contain modified bases such as inosine, methyl-5-deoxycytidine, deoxyuridine, dimethylamino-5-deoxyuridine, or diamino-2, 6-purine. Sugar or phosphate residues can also be modified or substituted. For example, a deoxyribose residue can be replaced by a polyamide (Nielsen *et al.*, Science (1991) 254:1497) and phosphate residues can be replaced by ester groups such as diphosphate, alkyl, arylphosphonate and phosphorothioate esters. In addition, the 2'-hydroxyl group on ribonucleotides can be modified by including, *e.g.*, alkyl groups.

Probes of the invention can be used in diagnostic tests, as capture or detection probes. Such capture probes can be conventionally immobilized on a solid support, directly or indirectly, by covalent means or by passive adsorption. A detection probe can be labelled by a detection marker selected from radioactive isotopes; enzymes such as peroxidase, alkaline phosphatase, and enzymes able to hydrolyze a chromogenic, fluorogenic, or luminescent substrate; compounds that are chromogenic, fluorogenic, or luminescent; nucleotide base analogs; and biotin.

Probes of the invention can be used in any conventional hybridization technique, such as dot blot (Maniatis *et al.*, Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), Southern blot (Southern, J. Mol. Biol. (1975) 98:503), northern blot (identical to Southern blot to the exception that RNA is used as a target), or the sandwich technique (Dunn *et al.*, Cell (1977) 12:23). The latter technique involves the use of a specific capture probe and/or a specific detection probe with nucleotide sequences that at least partially differ from each other.

A primer is usually a probe of about 10 to about 40 nucleotides that is used to initiate enzymatic polymerization of DNA in an amplification process (*e.g.*, PCR), in an elongation process, or in a reverse transcription method. In a diagnostic method involving PCR, primers can be labelled.

Thus, the invention also encompasses (i) a reagent containing a probe of the invention for detecting and/or identifying the presence of *Chlamydia* in a biological material; (ii) a method for detecting and/or identifying the presence of *Chlamydia* in a biological material, in which (a) a sample is recovered or derived from the biological material, (b) DNA or RNA is extracted from the material and denatured, and (c) exposed to a probe of the invention, for example, a capture, detection probe or both, under stringent hybridization conditions, such that hybridization is detected; and (iii) a method for detecting and/or identifying the presence of *Chlamydia* in a biological material, in which (a) a sample is recovered or derived from the biological material, (b) DNA is extracted therefrom, (c) the extracted DNA is primed with at least one, and preferably two, primers of the invention and amplified by polymerase chain reaction, and (d) the amplified DNA fragment is produced.

As previously mentioned, polypeptides that can be produced upon expression of the newly identified open reading frames are useful vaccine agents.

Therefore, a sixth aspect of the invention features a substantially purified polypeptide or polypeptide derivative having an amino acid sequence encoded by a polynucleotide of the invention.

A "substantially purified polypeptide" is defined as a polypeptide that is separated from the environment in which it naturally occurs and/or that is free of the majority of the polypeptides that are present in the environment in which it was synthesized. For example, a substantially purified polypeptide is free from cytoplasmic polypeptides. Those skilled in the art will understand that the polypeptides of the invention can be purified from a natural source, *i.e.*, a *Chlamydia* strain, or can be produced by recombinant means.

Homologous polypeptides or polypeptide derivatives encoded by polynucleotides of the invention can be screened for specific antigenicity by testing cross-reactivity with an antiserum raised against the polypeptide of reference having an amino acid sequence as shown in SEQ ID NOs:3 to 4. Briefly, a monospecific hyperimmune antiserum can be raised against a purified reference polypeptide as such or as a fusion polypeptide, for example, an expression product of MBP, GST, or His-tag systems or a synthetic peptide predicted to be antigenic. The homologous polypeptide or derivative screened for specific antigenicity can be produced as such or as a fusion polypeptide. In this latter case and if the antiserum is also raised against a fusion polypeptide, two different fusion systems are employed. Specific antigenicity can be determined

according to a number of methods, including Western blot (Towbin *et al.*, Proc. Natl. Acad. Sci. USA (1979) 76:4350), dot blot, and ELISA, as described below.

In a Western blot assay, the product to be screened, either as a purified preparation or a total *E. coli* extract, is submitted to SDS-Page electrophoresis as described by Laemmli (Nature (1970) 227:680). After transfer to a nitrocellulose membrane, the material is further incubated with the monospecific hyperimmune antiserum diluted in the range of dilutions from about 1:5 to about 1:5000, preferably from about 1:100 to about 1:500. Specific antigenicity is shown once a band corresponding to the product exhibits reactivity at any of the dilutions in the above range.

In an ELISA assay, the product to be screened is preferably used as the coating antigen. A purified preparation is preferred, although a whole cell extract can also be used. Briefly, about 100 μ l of a preparation at about 10 μ g protein/ml are distributed into wells of a 96-well polycarbonate ELISA plate. The plate is incubated for 2 hours at 37°C then overnight at 4°C. The plate is washed with phosphate buffer saline (PBS) containing 0.05% Tween 20 (PBS/Tween buffer). The wells are saturated with 250 μ l PBS containing 1% bovine serum albumin (BSA) to prevent non-specific antibody binding. After 1 hour incubation at 37°C, the plate is washed with PBS/Tween buffer. The antiserum is serially diluted in PBS/Tween buffer containing 0.5% BSA. 100 μ l of dilutions are added per well. The plate is incubated for 90 minutes at 37°C, washed and evaluated according to standard procedures. For example, a goat anti-rabbit peroxidase conjugate is added to the wells when specific antibodies were raised in rabbits. Incubation is carried out for 90 minutes at 37°C and the plate is washed. The reaction is developed with the appropriate substrate and the reaction is measured by colorimetry (absorbance measured spectrophotometrically). Under the above experimental conditions, a positive reaction is shown by O.D. values greater than a non immune control serum.

In a dot blot assay, a purified product is preferred, although a whole cell extract can also be used. Briefly, a solution of the product at about 100 μ g/ml is serially two-fold diluted in 50 mM Tris-HCl (pH 7.5). 100 μ l of each dilution are applied to a nitrocellulose membrane 0.45 μ m set in a 96-well dot blot apparatus (Biorad). The buffer is removed by applying vacuum to the system. Wells are washed by addition of 50 mM Tris-HCl (pH 7.5) and the membrane is air-dried. The membrane is saturated in blocking buffer (50 mM Tris-HCl (pH 7.5) 0.15 M NaCl, 10 g/L skim milk) and incubated with an antiserum dilution from about 1:50 to about 1:5000, preferably about 1:500. The reaction is revealed according to standard procedures. For example,

a goat anti-rabbit peroxidase conjugate is added to the wells when rabbit antibodies are used. Incubation is carried out 90 minutes at 37°C and the blot is washed. The reaction is developed with the appropriate substrate and stopped. The reaction is measured visually by the appearance of a colored spot, e.g., by colorimetry. Under the above experimental conditions, a positive
5 reaction is shown once a colored spot is associated with a dilution of at least about 1:5, preferably of at least about 1:500.

Therapeutic or prophylactic efficacy of a polypeptide or derivative of the invention can be evaluated as described below.

According to a seventh aspect of the invention, there is provided (i) a composition of
10 matter containing a polypeptide of the invention together with a diluent or carrier; in particular, (ii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a polypeptide of the invention; (iii) a method for inducing an immune response against *Chlamydia* in a mammal, by administering to the mammal an immunogenically effective amount
15 of a polypeptide of the invention to elicit an immune response, e.g., a protective immune response to *Chlamydia*; and particularly, (iv) a method for preventing and/or treating a *Chlamydia* (e.g., *C. trachomatis*, *C. psittaci*, *C. pneumoniae*, or *C. pecorum*) infection, by administering a prophylactic or therapeutic amount of a polypeptide of the invention to an individual in need. Additionally, the seventh aspect of the invention encompasses the use of a polypeptide of the invention in the preparation of a medicament for preventing and/or treating
20 *Chlamydia* infection.

The immunogenic compositions of the invention can be administered by any conventional route in use in the vaccine field, in particular to a mucosal (e.g., ocular, intranasal, pulmonary, oral, gastric, intestinal, rectal, vaginal, or urinary tract) surface or *via* the parenteral (e.g., subcutaneous, intradermal, intramuscular, intravenous, or intraperitoneal) route. The
25 choice of the administration route depends upon a number of parameters, such as the adjuvant associated with the polypeptide. For example, if a mucosal adjuvant is used, the intranasal or oral route will be preferred and if a lipid formulation or an aluminum compound is used, the parenteral route will be preferred. In the latter case, the sub-cutaneous or intramuscular route is most preferred. The choice can also depend upon the nature of the vaccine agent. For example,
30 a polypeptide of the invention fused to CTB or LTB will be best administered to a mucosal surface.

A composition of the invention can contain one or several polypeptides or derivatives of the invention. It can also contain at least one additional *Chlamydia* antigen, or a subunit, fragment, homolog, mutant, or derivative thereof.

For use in a composition of the invention, a polypeptide or derivative thereof can be formulated into or with liposomes, preferably neutral or anionic liposomes, microspheres, ISCOMS, or virus-like-particles (VLPs) to facilitate delivery and/or enhance the immune response. These compounds are readily available to one skilled in the art; for example, see Liposomes: A Practical Approach (*supra*).

Adjuvants other than liposomes and the like can also be used and are known in the art. A appropriate selection can conventionally be made by those skilled in the art, for example, from the list provided below.

Administration can be achieved in a single dose or repeated as necessary at intervals as can be determined by one skilled in the art. For example, a priming dose can be followed by three booster doses at weekly or monthly intervals. An appropriate dose depends on various parameters including the recipient (*e.g.*, adult or infant), the particular vaccine antigen, the route and frequency of administration, the presence/absence or type of adjuvant, and the desired effect (*e.g.*, protection and/or treatment), as can be determined by one skilled in the art. In general, a vaccine antigen of the invention can be administered by a mucosal route in an amount from about 10 µg to about 500 mg, preferably from about 1 mg to about 200 mg. For the parenteral route of administration, the dose usually should not exceed about 1 mg, preferably about 100 µg.

When used as vaccine agents, polynucleotides and polypeptides of the invention can be used sequentially as part of a multistep immunization process. For example, a mammal can be initially primed with a vaccine vector of the invention such as a pox virus, *e.g.*, via the parenteral route, and then boosted twice with the polypeptide encoded by the vaccine vector, *e.g.*, via the mucosal route. In another example, liposomes associated with a polypeptide or derivative of the invention can also be used for priming, with boosting being carried out mucosally using a soluble polypeptide or derivative of the invention in combination with a mucosal adjuvant (*e.g.*, LT).

A polypeptide derivative of the invention is also useful as a diagnostic reagent for detecting the presence of anti-*Chlamydia* antibodies, *e.g.*, in a blood sample. Such polypeptides are about 5 to about 80, preferably about 10 to about 50 amino acids in length and can be labeled

or unlabeled, depending upon the diagnostic method. Diagnostic methods involving such a reagent are described below.

Upon expression of a DNA molecule of the invention, a polypeptide or polypeptide derivative is produced and can be purified using known laboratory techniques. For example, the polypeptide or polypeptide derivative can be produced as a fusion protein containing a fused tail that facilitates purification. The fusion product can be used to immunize a small mammal, *e.g.*, a mouse or a rabbit, in order to raise antibodies against the polypeptide or polypeptide derivative (monospecific antibodies). The eighth aspect of the invention thus provides a monospecific antibody that binds to a polypeptide or polypeptide derivative of the invention.

By "monospecific antibody" is meant an antibody that is capable of reacting with a unique naturally-occurring *Chlamydia* polypeptide. An antibody of the invention can be polyclonal or monoclonal. Monospecific antibodies can be recombinant, *e.g.*, chimeric (*e.g.*, constituted by a variable region of murine origin associated with a human constant region), humanized (a human immunoglobulin constant backbone together with hypervariable region of animal, *e.g.*, murine, origin), and/or single chain. Both polyclonal and monospecific antibodies can also be in the form of immunoglobulin fragments, *e.g.*, F(ab)'2 or Fab fragments. The antibodies of the invention can be of any isotype, *e.g.*, IgG or IgA, and polyclonal antibodies can be of a single isotype or can contain a mixture of isotypes.

The antibodies of the invention, which are raised to a polypeptide or polypeptide derivative of the invention, can be produced and identified using standard immunological assays, *e.g.*, Western blot analysis, dot blot assay, or ELISA (see, *e.g.*, Coligan *et al.*, Current Protocols in Immunology (1994) John Wiley & Sons, Inc., New York, NY). The antibodies can be used in diagnostic methods to detect the presence of a *Chlamydia* antigen in a sample, such as a biological sample. The antibodies can also be used in affinity chromatography methods for purifying a polypeptide or polypeptide derivative of the invention. As is discussed further below, such antibodies can be used in prophylactic and therapeutic passive immunization methods.

Accordingly, a ninth aspect of the invention provides (i) a reagent for detecting the presence of *Chlamydia* in a biological sample that contains an antibody, polypeptide, or polypeptide derivative of the invention; and (ii) a diagnostic method for detecting the presence of *Chlamydia* in a biological sample, by contacting the biological sample with an antibody, a

polypeptide, or a polypeptide derivative of the invention, such that an immune complex is formed, and by detecting such complex to indicate the presence of *Chlamydia* in the sample or the organism from which the sample is derived.

Those skilled in the art will understand that the immune complex is formed between a component of the sample and the antibody, polypeptide, or polypeptide derivative, whichever is used, and that any unbound material can be removed prior to detecting the complex. As can be easily understood, a polypeptide reagent is useful for detecting the presence of anti-*Chlamydia* antibodies in a sample, e.g., a blood sample, while an antibody of the invention can be used for screening a sample, such as a gastric extract or biopsy, for the presence of *Chlamydia* polypeptides.

For use in diagnostic applications, the reagent (i.e., the antibody, polypeptide, or polypeptide derivative of the invention) can be in a free state or immobilized on a solid support, such as a tube, a bead, or any other conventional support used in the field. Immobilization can be achieved using direct or indirect means. Direct means include passive adsorption (non-covalent binding) or covalent binding between the support and the reagent. By "indirect means" is meant that an anti-reagent compound that interacts with a reagent is first attached to the solid support. For example, if a polypeptide reagent is used, an antibody that binds to it can serve as an anti-reagent, provided that it binds to an epitope that is not involved in the recognition of antibodies in biological samples. Indirect means can also employ a ligand-receptor system, for example, a molecule such as a vitamin can be grafted onto the polypeptide reagent and the corresponding receptor can be immobilized on the solid phase. This is illustrated by the biotin-streptavidin system. Alternatively, indirect means can be used, e.g., by adding to the reagent a peptide tail, chemically or by genetic engineering, and immobilizing the grafted or fused product by passive adsorption or covalent linkage of the peptide tail.

According to a tenth aspect of the invention, there is provided a process for purifying, from a biological sample, a polypeptide or polypeptide derivative of the invention, which involves carrying out antibody-based affinity chromatography with the biological sample, wherein the antibody is a monospecific antibody of the invention.

For use in a purification process of the invention, the antibody can be polyclonal or monospecific, and preferably is of the IgG type. Purified IgGs can be prepared from an antiserum using standard methods (see, e.g., Coligan *et al.*, *supra*). Conventional

chromatography supports, as well as standard methods for grafting antibodies, are disclosed in, e.g., Antibodies: A Laboratory Manual, D. Lane, E. Harlow, Eds. (1988).

Briefly, a biological sample, such as an *C. pneumoniae* extract, preferably in a buffer solution, is applied to a chromatography material, preferably equilibrated with the buffer used to dilute the biological sample so that the polypeptide or polypeptide derivative of the invention (i.e., the antigen) is allowed to adsorb onto the material. The chromatography material, such as a gel or a resin coupled to an antibody of the invention, can be in batch form or in a column. The unbound components are washed off and the antigen is then eluted with an appropriate elution buffer, such as a glycine buffer or a buffer containing a chaotropic agent, e.g., guanidine HCl, or high salt concentration (e.g., 3 M MgCl₂). Eluted fractions are recovered and the presence of the antigen is detected, e.g., by measuring the absorbance at 280 nm.

An antibody of the invention can be screened for therapeutic efficacy as described as follows. According to an eleventh aspect of the invention, there is provided (i) a composition of matter containing a monospecific antibody of the invention, together with a diluent or carrier; (ii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a monospecific antibody of the invention, and (iii) a method for treating or preventing a *Chlamydia* (e.g., *C. trachomatis*, *C. psittaci*, *C. pneumoniae* or *C. pecorum*) infection, by administering a therapeutic or prophylactic amount of a monospecific antibody of the invention to an individual in need. Additionally, the eleventh aspect of the invention encompasses the use of a monospecific antibody of the invention in the preparation of a medicament for treating or preventing *Chlamydia* infection.

To this end, the monospecific antibody can be polyclonal or monoclonal, preferably of the IgA isotype (predominantly). In passive immunization, the antibody can be administered to a mucosal surface of a mammal, e.g., the gastric mucosa, e.g., orally or intragastrically, advantageously, in the presence of a bicarbonate buffer. Alternatively, systemic administration, not requiring a bicarbonate buffer, can be carried out. A monospecific antibody of the invention can be administered as a single active component or as a mixture with at least one monospecific antibody specific for a different *Chlamydia* polypeptide. The amount of antibody and the particular regimen used can be readily determined by one skilled in the art. For example, daily administration of about 100 to 1,000 mg of antibodies over one week, or three doses per day of

about 100 to 1,000 mg of antibodies over two or three days, can be an effective regimens for most purposes.

Therapeutic or prophylactic efficacy can be evaluated using standard methods in the art, e.g., by measuring induction of a mucosal immune response or induction of protective and/or therapeutic immunity, using, e.g., the *C. pneumoniae* mouse model . Those skilled in the art will recognize that the *C. pneumoniae* strain of the model can be replaced with another *Chlamydia* strain. For example, the efficacy of DNA molecules and polypeptides from *C. pneumoniae* is preferably evaluated in a mouse model using an *C. pneumoniae* strain. Protection can be determined by comparing the degree of *Chlamydia* infection to that of a control group. Protection is shown when infection is reduced by comparison to the control group. Such an evaluation can be made for polynucleotides, vaccine vectors, polypeptides and derivatives thereof, as well as antibodies of the invention.

Adjuvants useful in any of the vaccine compositions described above are as follows.

Adjuvants for parenteral administration include aluminum compounds, such as aluminum hydroxide, aluminum phosphate, and aluminum hydroxy phosphate. The antigen can be precipitated with, or adsorbed onto, the aluminum compound according to standard protocols. Other adjuvants, such as RIBI (ImmunoChem, Hamilton, MT), can be used in parenteral administration.

Adjuvants for mucosal administration include bacterial toxins, e.g., the cholera toxin (CT), the *E. coli* heat-labile toxin (LT), the *Clostridium difficile* toxin A and the pertussis toxin (PT), or combinations, subunits, toxoids, or mutants thereof. For example, a purified preparation of native cholera toxin subunit B (CTB) can be of use. Fragments, homologs, derivatives, and fusions to any of these toxins are also suitable, provided that they retain adjuvant activity. Preferably, a mutant having reduced toxicity is used. Suitable mutants are described, e.g., in WO 95/17211 (Arg-7-Lys CT mutant), WO 96/6627 (Arg-192-Gly LT mutant), and WO 95/34323 (Arg-9-Lys and Glu-129-Gly PT mutant). Additional LT mutants that can be used in the methods and compositions of the invention include, e.g., Ser-63-Lys, Ala-69-Gly, Glu-110-Asp, and Glu-112-Asp mutants. Other adjuvants, such as a bacterial monophosphoryl lipid A (MPLA) of, e.g., *E. coli*, *Salmonella minnesota*, *Salmonella typhimurium*, or *Shigella flexneri*; saponins, or polylactide glycolide (PLGA) microspheres, can also be used in mucosal administration.

Adjuvants useful for both mucosal and parenteral administrations include polyphosphazene (WO 95/2415), DC-chol (3 b-(N-(N',N'-dimethyl aminomethane)-carbamoyl) cholesterol; U.S. Patent No. 5,283,185 and WO 96/14831) and QS-21 (WO 88/9336).

Any pharmaceutical composition of the invention, containing a polynucleotide, a polypeptide, a polypeptide derivative, or an antibody of the invention, can be manufactured in a conventional manner. In particular, it can be formulated with a pharmaceutically acceptable diluent or carrier, e.g., water or a saline solution such as phosphate buffer saline. In general, a diluent or carrier can be selected on the basis of the mode and route of administration, and standard pharmaceutical practice. Suitable pharmaceutical carriers or diluents, as well as pharmaceutical necessities for their use in pharmaceutical formulations, are described in *Remington's Pharmaceutical Sciences*, a standard reference text in this field and in the USP/NF.

The invention also includes methods in which *Chlamydia* infection, are treated by oral administration of a *Chlamydia* polypeptide of the invention and a mucosal adjuvant, in combination with an antibiotic, an antacid, sucralfate, or a combination thereof. Examples of such compounds that can be administered with the vaccine antigen and the adjuvant are antibiotics, including, e.g., macrolides, tetracyclines, and derivatives thereof (specific examples of antibiotics that can be used include azithromycin or doxycyclin or immunomodulators such as cytokines or steroids. In addition, compounds containing more than one of the above-listed components coupled together, can be used. The invention also includes compositions for carrying out these methods, i.e., compositions containing a *Chlamydia* antigen (or antigens) of the invention, an adjuvant, and one or more of the above-listed compounds, in a pharmaceutically acceptable carrier or diluent.

Amounts of the above-listed compounds used in the methods and compositions of the invention can readily be determined by one skilled in the art. In addition, one skilled in the art can readily design treatment/immunization schedules. For example, the non-vaccine components can be administered on days 1-14, and the vaccine antigen + adjuvant can be administered on days 7, 14, 21, and 28.

REFERENCES

1. Grayston et al. (1995) Journal of Infectious Diseases 168:1231
2. Campos et al. (1995) Investigation of Ophthalmology and Visual Science 36:1477
3. Grayston et al (1990) Journal of Infectious Diseases 161:618
- 5 4. Marrie (1993) Clinical Infectious Diseases. 18:501
5. Wang et al (1986) Chlamydial infections. Cambridge University Press, Cambridge. p. 329
6. Saikku et al.(1988) Lancet;ii:983
7. Thom et al. (1992) JAMA 268:68
8. Linnanmaki et al. (1993), Circulation 87:1030
- 10 9. Saikku et al. (1992)Annals Internal Medicine 116:273
10. Melnick et al(1993) American Journal of Medicine 95:499
- 11 Shor et al. (1992) South African. Medical Journal 82:158
12. Kuo et al. (1993) Journal of Infectious Diseases 167:841
13. Kuo et al. (1993) Arteriosclerosis and Thrombosis 13:1500
- 15 14. Campbell et al (1995) Journal of Infectious Diseases 172:585
15. Chiu et al. Circulation, 1997 (In Press).
16. Ramirez et al (1996) Annals of Internal Medicine 125:979
17. Jackson et al. Abst. K121, p272, 36th ICAAC, 15-18 Sept. 1996, New Orleans.
18. Fong et al (1997) Journal of Clinical Microbiology 35:48
- 20 19. Hahn DL, et al. Evidence for Chlamydia pneumoniae infection in steroid-dependent asthma.
Ann Allergy Asthma Immunol. 1998 Jan; 80(1): 45-49.
20. Hahn DL, et al. Association of Chlamydia pneumoniae IgA antibodies with recently
symptomatic asthma. Epidemiol Infect. 1996 Dec; 117(3): 513-517.
21. Bjornsson E, et al. Serology of chlamydia in relation to asthma and bronchial
25 hyperresponsiveness. Scand J Infect Dis. 1996; 28(1): 63-69.
22. Hahn DL. Treatment of *Chlamydia pneumoniae* infection in adult asthma: a before-after trial.
J Fam Pract. 1995 Oct; 41(4): 345-351.
23. Allegra L, et al. Acute exacerbations of asthma in adults: role of Chlamydia pneumoniae
infection. Eur Respir J. 1994 Dec; 7(12): 2165-2168.
- 30 24. Hahn DL, et al. Association of Chlamydia pneumoniae (strain TWAR) infection with
wheezing, asthmatic bronchitis, and adult-onset asthma. JAMA. 1991 Jul 10; 266(2): 225-230.

25. Pal *et al.* (1996) *Infection and Immunity* 64:5341
26. Jones *et al.* (1995) *Vaccine* 13:715
27. Igietsemes *et al.* (1993) *Immunology* 5:317
28. Igietseme *et al.* (1993) *Regional Immunology* 5:317
- 5 29. Magee *et al.* (1993) *Regional Immunology* 5: 305
30. Landers *et al.* (1991) *Infection & Immunity* 59:3774
31. Magee *et al.* (1995) *Infection & Immunity* 63:516
32. Cotter *et al.* (1995) *Infection and Immunity* 63:4704
33. Campbell *et al.* (1990) *Infection and Immunity* 58:93
- 10 34. McCafferty *et al.* (1995) *Infection and Immunity* 63:2387-9.
35. Knudsen *et al.* (1996) Third Meeting of the European Society for Chlamydia Research, Vienna
36. Wiedmann-Al-Ahmad M, *et al.* Reactions of polyclonal and neutralizing anti-p54 monoclonal antibodies with an isolated, species-specific 54-kilodalton protein of *Chlamydia pneumoniae*. *Clin Diagn Lab Immunol.* 1997 Nov; 4(6): 700-704
- 15 37. Hughes *et al.*, 1992. *Infect. Immun.* 60(9):3497,
38. Dion *et al.*, 1990. *Virology* 179:474-477,
39. Snijders *et al.*, 1991. *J. Gen. Virol.* 72:557-565,
40. Langeveld *et al.*, *Vaccine* 12(15):1473-1480, 1994
41. Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons Inc., 1994
- 20 42. Kunkel *et al.* *Proc. Natl. Acad. Sci. USA* (1985) 82:448
43. Silhavy *et al.* *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory Press, 1984
44. Davis *et al.* *A Manual for Genetic Engineering: Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory Press, 1980)
45. Casey & Davidson, *Nucl. Acid Res.* (1977) 4:1539
- 25 46. Cagnon *et al.*, *Protein Engineering* (1991) 4(7):843
47. Takase *et al.*, *J. Bact.* (1987) 169:5692

Figure 1

agttaaagat gacaaaacag ctgtcaagaa tttttatctt gactctctga gttttctatt 60

ttatatgacg caagtaagaa ttttaataata aagtgggttt atg aaa tcg caa ttt 115
Met Lys Ser Gln Phe
1 5

tcc tgg tta gtg ctc tct tcg aca ttg gca tgt ttt act agt tgt tcc 163
Ser Trp Leu Val Leu Ser Ser Thr Leu Ala Cys Phe Thr Ser Cys Ser
10 15 20

act gtt ttt gct gca act gct gaa aat ata ggc ccc tct gat agc ttt 211
Thr Val Phe Ala Ala Thr Ala Glu Asn Ile Gly Pro Ser Asp Ser Phe
25 30 35

gac gga agt act aac aca ggc acc tat act cct aaa aat acg act act 259
Asp Gly Ser Thr Asn Thr Gly Thr Tyr Thr Pro Lys Asn Thr Thr Thr
Gly Thr Tyr Thr Pro Lys Asn Thr Thr Thr
40 45 50

gga ata gac tat act ctg aca gga gat ata act ctg caa aac ctt ggg 307
Gly Ile Asp Tyr Thr Leu Thr Gly Asp Ile Thr Leu Gln Asn Leu Gly
Gly Ile Asp Tyr Thr Leu Thr Gly Asp Ile Thr Leu Gln Asn Leu Gly
55 60 65

gat tcg gca gct tta acg aag ggt tgt ttt tct gac act acg gaa tct 355
Asp Ser Ala Ala Leu Thr Lys Gly Cys Phe Ser Asp Thr Thr Glu Ser
Asp Ser Ala Ala Leu Thr Lys Gly Cys Phe Ser Asp Thr Thr Glu Ser
70 75 80 85

tta agc ttt gcc ggt aag ggg tac tca ctt tct ttt tta aat att aag 403
Leu Ser Phe Ala Gly Lys Gly Tyr Ser Leu Ser Phe Leu Asn Ile Lys
Leu Ser Phe Ala Gly Lys Gly Tyr Ser Leu Ser Phe Leu Asn Ile Lys
90 95 100

tct agt gct gaa ggc gca gcc ctt tct gtt aca act gat aaa aat ctg 451
Ser Ser Ala Glu Gly Ala Ala Leu Ser Val Thr Thr Asp Lys Asn Leu
Ser Ser Ala Glu Gly Ala Ala Leu Ser Val Thr Thr Asp Lys Asn Leu
105 110 115

tcg cta aca gga ttt tcg agt ctt act ttc tta gcg gcc cca tca tcg 499
Ser Leu Thr Gly Phe Ser Ser Leu Thr Phe Leu Ala Ala Pro Ser Ser
Ser Leu Thr Gly Phe Ser Ser Leu Thr Phe Leu Ala Ala Pro Ser Ser
120 125 130

gta atc aca acc ccc tca gga aaa ggt gca gtt aaa tgt gga ggg gat 547
Val Ile Thr Thr Pro Ser Gly Lys Gly Ala Val Lys Cys Gly Gly Asp
Val Ile Thr Thr Pro Ser Gly Lys Gly Ala Val Lys Cys Gly Gly Asp
135 140 145

ctt aca ttt gat aac aat gga act att tta ttt aaa caa gat tac tgt 595
Leu Thr Phe Asp Asn Asn Gly Thr Ile Leu Phe Lys Gln Asp Tyr Cys
Leu Thr Phe Asp Asn Asn Gly Thr Ile Leu Phe Lys Gln Asp Tyr Cys
150 155 160 165

gag gaa aat ggc gga gcc att tct acc aag aat ctt tct ttg aaa aac 643
Glu Glu Asn Gly Gly Ala Ile Ser Thr Lys Asn Leu Ser Leu Lys Asn
Glu Glu Asn Gly Gly Ala Ile Ser Thr Lys Asn Leu Ser Leu Lys Asn
170 175 180

agc acg gga tcg att tct ttt gaa ggg aat aaa tcg agc gca aca ggg 691
Ser Thr Gly Ser Ile Ser Phe Glu Gly Asn Lys Ser Ser Ala Thr Gly

Ser	Thr	Gly	Ser	Ile	Ser	Phe	Glu	Gly	Asn	Lys	Ser	Ser	Ala	Thr	Gly		
			185					190					195				
aaa	aaa	ggt	ggg	gct	att	tgt	gct	act	ggt	act	gta	gat	att	aca	aat	739	
Lys	Lys	Gly	Gly	Ala	Ile	Cys	Ala	Thr	Gly	Thr	Val	Asp	Ile	Thr	Asn		
Lys	Lys	Gly	Gly	Ala	Ile	Cys	Ala	Thr	Gly	Thr	Val	Asp	Ile	Thr	Asn		
		200					205					210					
aat	acg	gct	cct	acc	ctc	ttc	tcg	aac	aat	att	gct	gaa	gct	gca	ggt	787	
Asn	Thr	Ala	Pro	Thr	Leu	Phe	Ser	Asn	Asn	Ile	Ala	Glu	Ala	Ala	Gly		
Asn	Thr	Ala	Pro	Thr	Leu	Phe	Ser	Asn	Asn	Ile	Ala	Glu	Ala	Ala	Gly		
		215				220					225						
gga	gct	ata	aat	agc	aca	gga	aac	tgt	aca	att	aca	ggg	aat	acg	tct	835	
Gly	Ala	Ile	Asn	Ser	Thr	Gly	Asn	Cys	Thr	Ile	Thr	Gly	Asn	Thr	Ser		
Gly	Ala	Ile	Asn	Ser	Thr	Gly	Asn	Cys	Thr	Ile	Thr	Gly	Asn	Thr	Ser		
		230				235				240					245		
ctt	gta	ttt	tct	gaa	aat	agt	gtg	aca	gcg	acc	gca	gga	aat	gga	gga	883	
Leu	Val	Phe	Ser	Glu	Asn	Ser	Val	Thr	Ala	Thr	Ala	Gly	Asn	Gly	Gly		
Leu	Val	Phe	Ser	Glu	Asn	Ser	Val	Thr	Ala	Thr	Ala	Gly	Asn	Gly	Gly		
				250					255					260			
gct	ctt	tct	gga	gat	gcc	gat	gtt	acc	ata	tct	ggg	aat	cag	agt	gta	931	
Ala	Leu	Ser	Gly	Asp	Ala	Asp	Val	Thr	Ile	Ser	Gly	Asn	Gln	Ser	Val		
Ala	Leu	Ser	Gly	Asp	Ala	Asp	Val	Thr	Ile	Ser	Gly	Asn	Gln	Ser	Val		
			265					270					275				
act	ttc	tca	gga	aac	caa	gct	gta	gct	aat	ggc	gga	gcc	att	tat	gct	979	
Thr	Phe	Ser	Gly	Asn	Gln	Ala	Val	Ala	Asn	Gly	Gly	Ala	Ile	Tyr	Ala		
Thr	Phe	Ser	Gly	Asn	Gln	Ala	Val	Ala	Asn	Gly	Gly	Ala	Ile	Tyr	Ala		
		280					285					290					
aag	aag	ctt	aca	ctg	gct	tcc	ggg	ggg	ggg	ggg	ggg	aat	ccc	ttt	tct	1027	
Lys	Lys	Leu	Thr	Leu	Ala	Ser	Gly	Gly	Gly	Gly	Gly	Asn	Pro	Phe	Ser		
Lys	Lys	Leu	Thr	Leu	Ala	Ser	Gly	Gly	Gly	Gly	Gly	Asn	Pro	Phe	Ser		
		295				300					305						
aac	aat	ata	gtc	caa	ggg	acc	act	gca	ggt	aat	ggt	gga	gcc	att	tct	1075	
Asn	Asn	Ile	Val	Gln	Gly	Thr	Thr	Ala	Gly	Asn	Gly	Gly	Ala	Ile	Ser		
Asn	Asn	Ile	Val	Gln	Gly	Thr	Thr	Ala	Gly	Asn	Gly	Gly	Ala	Ile	Ser		
		310			315					320					325		
ata	ctg	gca	gct	gga	gag	tgt	agt	ctt	ttc	agc	gaa	gca	ggg	gac	cat	1123	
Ile	Leu	Ala	Ala	Gly	Glu	Cys	Ser	Leu	Phe	Ser	Glu	Ala	Gly	Asp	His		
Ile	Leu	Ala	Ala	Gly	Glu	Cys	Ser	Leu	Phe	Ser	Glu	Ala	Gly	Asp	His		
				330					335					340			
tac	ctt	aat	ggg	aat	gcc	att	gtt	gca	act	aca	cca	caa	act	aca	aaa	1171	
Tyr	Leu	Asn	Gly	Asn	Ala	Ile	Val	Ala	Thr	Thr	Pro	Gln	Thr	Thr	Lys		
Tyr	Leu	Asn	Gly	Asn	Ala	Ile	Val	Ala	Thr	Thr	Pro	Gln	Thr	Thr	Lys		
			345						350				355				
aga	aat	tct	att	gac	ata	gga	tct	act	ggc	aaa	gat	cac	gaa	tta	cggt	1219	
Arg	Asn	Ser	Ile	Asp	Ile	Gly	Ser	Thr	Gly	Lys	Asp	His	Glu	Leu	Arg		
Arg	Asn	Ser	Ile	Asp	Ile	Gly	Ser	Thr	Gly	Lys	Asp	His	Glu	Leu	Arg		
		360					365					370					
gca	ata	tct	ggg	cat	agc	atc	ttt	ttc	tac	gat	ccg	att	act	gct	aat	1267	
Ala	Ile	Ser	Gly	His	Ser	Ile	Phe	Phe	Tyr	Asp	Pro	Ile	Thr	Ala	Asn		
Ala	Ile	Ser	Gly	His	Ser	Ile	Phe	Phe	Tyr	Asp	Pro	Ile	Thr	Ala	Asn		
		375				380					385						
acg	gct	gcg	gat	tct	aca	gat	act	tta	aat	ctc	aat	aag	gct	gat	gca	1315	

Thr Ala Ala Asp Ser Thr Asp Thr Leu Asn Leu Asn Lys Ala Asp Ala
 Thr Ala Ala Asp Ser Thr Asp Thr Leu Asn Leu Asn Lys Ala Asp Ala
 390 395 400 405

ggt aat agt aca gat tat agt ggg tcg att gtt ttt tct ggt gaa aag 1363
 Gly Asn Ser Thr Asp Tyr Ser Gly Ser Ile Val Phe Ser Gly Glu Lys
 Gly Asn Ser Thr Asp Tyr Ser Gly Ser Ile Val Phe Ser Gly Glu Lys
 410 415 420

ctc tct gaa gat gaa gca aaa gtt gca gac aac ctc act tct acg ctg 1411
 Leu Ser Glu Asp Glu Ala Lys Val Ala Asp Asn Leu Thr Ser Thr Leu
 Leu Ser Glu Asp Glu Ala Lys Val Ala Asp Asn Leu Thr Ser Thr Leu
 425 430 435

aag cag cct gta act cta act gca gga aat tta gta ctt aaa cgt ggt 1459
 Lys Gln Pro Val Thr Leu Thr Ala Gly Asn Leu Val Leu Lys Arg Gly
 Lys Gln Pro Val Thr Leu Thr Ala Gly Asn Leu Val Leu Lys Arg Gly
 440 445 450

gtc act ctc gat acg aaa ggc ttt act cag acc gcg ggt tcc tct gtt 1507
 Val Thr Leu Asp Thr Lys Gly Phe Thr Gln Thr Ala Gly Ser Ser Val
 Val Thr Leu Asp Thr Lys Gly Phe Thr Gln Thr Ala Gly Ser Ser Val
 455 460 465

att atg gat gcg ggc aca acg tta aaa gca agt aca gag gag gtc act 1555
 Ile Met Asp Ala Gly Thr Thr Leu Lys Ala Ser Thr Glu Glu Val Thr
 Ile Met Asp Ala Gly Thr Thr Leu Lys Ala Ser Thr Glu Glu Val Thr
 470 475 480 485

tta aca ggt ctt tcc att cct gta gac tct tta ggc gag ggt aag aaa 1603
 Leu Thr Gly Leu Ser Ile Pro Val Asp Ser Leu Gly Glu Gly Lys Lys
 Leu Thr Gly Leu Ser Ile Pro Val Asp Ser Leu Gly Glu Gly Lys Lys
 490 495 500

gtt gta att gct gct tct gca gca agt aaa aat gta gcc ctt agt ggt 1651
 Val Val Ile Ala Ala Ser Ala Ala Ser Lys Asn Val Ala Leu Ser Gly
 Val Val Ile Ala Ala Ser Ala Ala Ser Lys Asn Val Ala Leu Ser Gly
 505 510 515

ccg att ctt ctt ttg gat aac caa ggg aat gct tat gaa aat cac gac 1699
 Pro Ile Leu Leu Leu Asp Asn Gln Gly Asn Ala Tyr Glu Asn His Asp
 Pro Ile Leu Leu Leu Asp Asn Gln Gly Asn Ala Tyr Glu Asn His Asp
 520 525 530

tta gga aaa act caa gac ttt tca ttt gtg cag ctc tct gct ctg ggt 1747
 Leu Gly Lys Thr Gln Asp Phe Ser Phe Val Gln Leu Ser Ala Leu Gly
 Leu Gly Lys Thr Gln Asp Phe Ser Phe Val Gln Leu Ser Ala Leu Gly
 535 540 545

act gca aca act aca gat gtt cca gcg gtt cct aca gta gca act cct 1795
 Thr Ala Thr Thr Thr Asp Val Pro Ala Val Pro Thr Val Ala Thr Pro
 Thr Ala Thr Thr Thr Asp Val Pro Ala Val Pro Thr Val Ala Thr Pro
 550 555 560 565

acg cac tat ggg tat caa ggt act tgg gga atg act tgg gtt gat gat 1843
 Thr His Tyr Gly Tyr Gln Gly Thr Trp Gly Met Thr Trp Val Asp Asp
 Thr His Tyr Gly Tyr Gln Gly Thr Trp Gly Met Thr Trp Val Asp Asp
 570 575 580

acc gca agc act cca aag act aag aca gcg aca tta gct tgg acc aat 1891
 Thr Ala Ser Thr Pro Lys Thr Lys Thr Ala Thr Leu Ala Trp Thr Asn
 Thr Ala Ser Thr Pro Lys Thr Lys Thr Ala Thr Leu Ala Trp Thr Asn
 585 590 595

aca ggc tac ctt ccg aat cct gag cgt caa gga cct tta gtt cct aat 1939
 Thr Gly Tyr Leu Pro Asn Pro Glu Arg Gln Gly Pro Leu Val Pro Asn
 Thr Gly Tyr Leu Pro Asn Pro Glu Arg Gln Gly Pro Leu Val Pro Asn
 600 605 610

agc ctt tgg gga tct ttt tca gac atc caa gcg att caa ggt gtc ata 1987
 Ser Leu Trp Gly Ser Phe Ser Asp Ile Gln Ala Ile Gln Gly Val Ile
 Ser Leu Trp Gly Ser Phe Ser Asp Ile Gln Ala Ile Gln Gly Val Ile
 615 620 625

gag aga agt gct ttg act ctt tgt tca gat cga ggc ttc tgg gct gcg 2035
 Glu Arg Ser Ala Leu Thr Leu Cys Ser Asp Arg Gly Phe Trp Ala Ala
 Glu Arg Ser Ala Leu Thr Leu Cys Ser Asp Arg Gly Phe Trp Ala Ala
 630 635 640 645

gga gtc gcc aat ttc tta gat aaa gat aag aaa ggg gaa aaa cgc aaa 2083
 Gly Val Ala Asn Phe Leu Asp Lys Asp Lys Lys Gly Glu Lys Arg Lys
 Gly Val Ala Asn Phe Leu Asp Lys Asp Lys Lys Gly Glu Lys Arg Lys
 650 655 660

tac cgt cat aaa tct ggt gga tat gct atc gga ggt gca gcg caa act 2131
 Tyr Arg His Lys Ser Gly Gly Tyr Ala Ile Gly Gly Ala Ala Gln Thr
 Tyr Arg His Lys Ser Gly Gly Tyr Ala Ile Gly Gly Ala Ala Gln Thr
 665 670 675

tgt tct gaa aac tta att agc ttt gcc ttt tgc caa ctc ttt ggt agc 2179
 Cys Ser Glu Asn Leu Ile Ser Phe Ala Phe Cys Gln Leu Phe Gly Ser
 Cys Ser Glu Asn Leu Ile Ser Phe Ala Phe Cys Gln Leu Phe Gly Ser
 680 685 690

gat aaa gat ttc tta gtc gct aaa aat cat act gat acc tat gca gga 2227
 Asp Lys Asp Phe Leu Val Ala Lys Asn His Thr Asp Thr Tyr Ala Gly
 Asp Lys Asp Phe Leu Val Ala Lys Asn His Thr Asp Thr Tyr Ala Gly
 695 700 705

gcc ttc tat atc caa cac att aca gaa tgt agt ggg ttc ata ggt tgt 2275
 Ala Phe Tyr Ile Gln His Ile Thr Glu Cys Ser Gly Phe Ile Gly Cys
 Ala Phe Tyr Ile Gln His Ile Thr Glu Cys Ser Gly Phe Ile Gly Cys
 710 715 720 725

ctc tta gat aaa ctt cct ggc tct tgg agt cat aaa ccc ctc gtt tta 2323
 Leu Leu Asp Lys Leu Pro Gly Ser Trp Ser His Lys Pro Leu Val Leu
 Leu Leu Asp Lys Leu Pro Gly Ser Trp Ser His Lys Pro Leu Val Leu
 730 735 740

gaa ggg cag ctc gct tat agc cac gtc agt aat gat ctg aag aca aag 2371
 Glu Gly Gln Leu Ala Tyr Ser His Val Ser Asn Asp Leu Lys Thr Lys
 Glu Gly Gln Leu Ala Tyr Ser His Val Ser Asn Asp Leu Lys Thr Lys
 745 750 755

tat act gcg tat cct gag gtg aaa ggt tct tgg ggg aat aat gct ttt 2419
 Tyr Thr Ala Tyr Pro Glu Val Lys Gly Ser Trp Gly Asn Asn Ala Phe
 Tyr Thr Ala Tyr Pro Glu Val Lys Gly Ser Trp Gly Asn Asn Ala Phe
 760 765 770

aac atg atg ttg gga gct tct tct cat tct tat cct gaa tac ctg cat 2467
 Asn Met Met Leu Gly Ala Ser Ser His Ser Tyr Pro Glu Tyr Leu His
 Asn Met Met Leu Gly Ala Ser Ser His Ser Tyr Pro Glu Tyr Leu His
 775 780 785

tgt ttt gat acc tat gct cca tac atc aaa ctg aat ctg acc tat ata 2515
 Cys Phe Asp Thr Tyr Ala Pro Tyr Ile Lys Leu Asn Leu Thr Tyr Ile
 Cys Phe Asp Thr Tyr Ala Pro Tyr Ile Lys Leu Asn Leu Thr Tyr Ile
 790 795 800 805

cgt cag gac agc ttc tcg gag aaa ggt aca gaa gga aga tct ttt gat 2563
 Arg Gln Asp Ser Phe Ser Glu Lys Gly Thr Glu Gly Arg Ser Phe Asp
 Arg Gln Asp Ser Phe Ser Glu Lys Gly Thr Glu Gly Arg Ser Phe Asp
 810 815 820

gac agc aac ctc ttc aat tta tct ttg cct ata ggg gtg aag ttt gag 2611
 Asp Ser Asn Leu Phe Asn Leu Ser Leu Pro Ile Gly Val Lys Phe Glu
 Asp Ser Asn Leu Phe Asn Leu Ser Leu Pro Ile Gly Val Lys Phe Glu
 825 830 835

aag ttc tct gat tgt aat gac ttt tct tat gat ctg act tta tcc tat 2659
 Lys Phe Ser Asp Cys Asn Asp Phe Ser Tyr Asp Leu Thr Leu Ser Tyr
 Lys Phe Ser Asp Cys Asn Asp Phe Ser Tyr Asp Leu Thr Leu Ser Tyr
 840 845 850

gtt cct gat ctt atc cgc aat gat ccc aaa tgc act aca gca ctt gta 2707
 Val Pro Asp Leu Ile Arg Asn Asp Pro Lys Cys Thr Thr Ala Leu Val
 Val Pro Asp Leu Ile Arg Asn Asp Pro Lys Cys Thr Thr Ala Leu Val
 855 860 865

atc agc gga gcc tct tgg gaa act tat gcc aat aac tta gca cga cag 2755
 Ile Ser Gly Ala Ser Trp Glu Thr Tyr Ala Asn Asn Leu Ala Arg Gln
 Ile Ser Gly Ala Ser Trp Glu Thr Tyr Ala Asn Asn Leu Ala Arg Gln
 870 875 880 885

gcc ttg caa gtg cgt gca ggc agt cac tac gcc ttc tct cct atg ttt 2803
 Ala Leu Gln Val Arg Ala Gly Ser His Tyr Ala Phe Ser Pro Met Phe
 Ala Leu Gln Val Arg Ala Gly Ser His Tyr Ala Phe Ser Pro Met Phe
 890 895 900

gaa gtg ctc ggc cag ttt gtc ttt gaa gtt cgt gga tcc tca cgg att 2851
 Glu Val Leu Gly Gln Phe Val Phe Glu Val Arg Gly Ser Ser Arg Ile
 Glu Val Leu Gly Gln Phe Val Phe Glu Val Arg Gly Ser Ser Arg Ile
 905 910 915

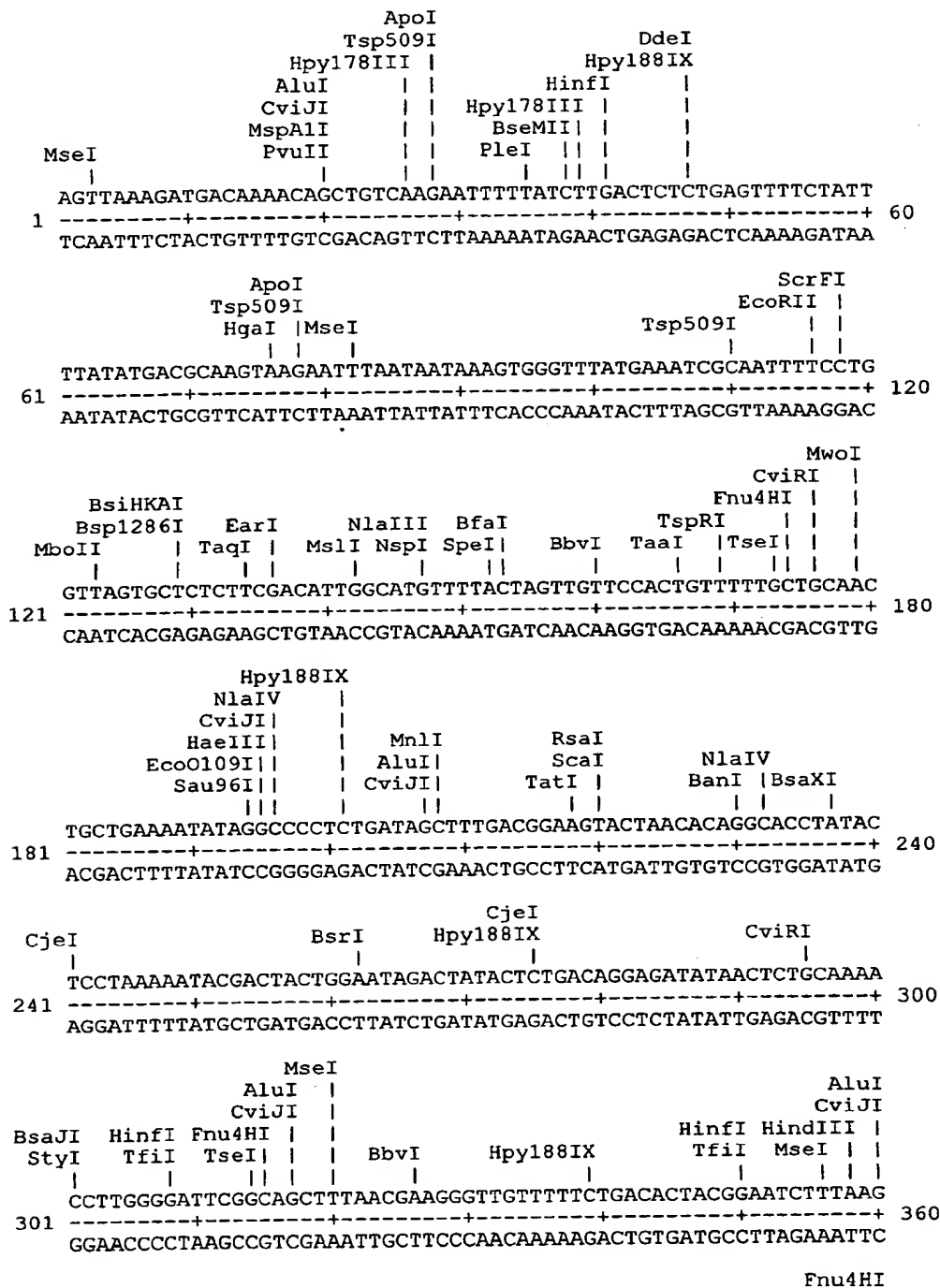
tat aat gta gat ctt ggg ggt aag ttc caa ttc taggagcgtc tctcatgtct 2904
 Tyr Asn Val Asp Leu Gly Gly Lys Phe Gln Phe
 Tyr Asn Val Asp Leu Gly Gly Lys Phe Gln Phe
 920 925

cagaaattct gagagagatc gcatttagga ttttcttaaa cacgac 2950

2563-2904
 2904-2950

Figure 2

Restriction enzyme analysis of CPN100635



```

      MspI      DraI  MseI      HhaI|
BsrFI|  BslI RsaI      MseI|SspI|  BfaI      MwoI  |
      ||      ||      ||      ||      ||      ||
CTTTGCCGGTAAGGGGTACTCACTTTCTTTTTTAAATATTAAGTCTAGTGCTGAAGGCGC
361 -----+-----+-----+-----+-----+-----+-----+-----+ 420
      GAAACGGCCATTCCCCATGAGTGAAAGAAAAAATTTATAATTCAGATCACGACTTCCGCG

      Eco57I
      BbvI|
CviJI  MaeIII|      BsaBI      HinfI      PleI
      |      ||      |      |      |
AGCCCTTTCTGTTACAACGTATAAAAAATCTGTCGCTAACAGGATTTTCGAGTCTTACTTT
421 -----+-----+-----+-----+-----+-----+-----+ 480
      TCGGGAAGACAATGTTGACTATTTTGTAGACAGCGATTGTCCTAAAAGCTCAGAATGAAA

      BccI
      NlaIV|
      CviJI|
      HaeIII|
      Sau96I|
      Fnu4HI|
      TauI|
      AciI|
CjeI  |||||  BslI
DdeI  |||||  |||||
      |||||  |||||
CTTAGCGGCCCCATCATCGGTAATCACAACCCCTCAGGAAAAGGTGCAGTTAAATGTGG
481 -----+-----+-----+-----+-----+-----+-----+ 540
      GAATCGCCGGGGTAGTAGCCATTAGTGTTGGGGGAGTCCTTTCCACGTCAATTACACC

      BspI
      DpnI
      BstYI|
      Sau3AI|  AlwI
      ||      |
AGGGGATCTTACATTTGATAACAATGGAACATTTTTATTTAAACAAGATTACTGTGAGGA
541 -----+-----+-----+-----+-----+-----+-----+ 600
      TCCCCTAGAATGTAAACTATTGTTACCTTGATAAAATAAATTTGTTCTAATGACACTCCT

      CviJI
      NlaIV|
      EciI  ||
      AciI  ||
      ||      ||
AAATGGCGGAGCCATTTCTACCAAGAATCTTTCTTTGAAAAACAGCAGGGGATCGATTTC
601 -----+-----+-----+-----+-----+-----+-----+ 660
      TTTACCGCCTCGGTAAAGATGGTCTTAGAAAGAACTTTTTGTCGTGCCCTAGCTAAAG

      HhaI
      CjePI|
      TaqI  ||
      ||      ||
TTTGAAGGGAATAAATCGAGCGCAACAGGGAAAAAAGGTGGGGCTATTTGTGCTACTGG
661 -----+-----+-----+-----+-----+-----+-----+ 720
      AAAACTTCCCTTATTTAGCTCGCGTTGTCCCTTTTTTCCACCCGATAAACACGATGACC

      BspMI
      SspI|
      BbvI|  Fnu4HI
      MnlI|  |  SfcI
      EarI|  |  AluI|
TaaI  |  MboII  Hpy178III|  |  CviJI|
SfcI|

```


39

40

41

MwoI || | DdeI MboI || | BsaJI
 TseI || | BbvI CviJI | Sau96I | | StyI
 || | || | || | || |
 1621 TGCAGCAAGTAAAAATGTAGCCCTTAGTGGTCCGATTCTTCTTTTGGATAACCAAGGGAA 1680
 -----+-----+-----+-----+-----+
 ACGTCGTTTCATTTTACATCGGGAATCACCAGGCTAAGAAGAAAACCTATTGGTTCCTT

AluI
 CviJI
 Fnu4HI |
 DdeI CviRI |
 Bce83I | Hpy178III SmlI |
 BsmI Hpy178III | | TseI |
 | | | || |
 1681 TGCTTATGAAAATCAGACTTAGGAAAACTCAAGACTTTTCATTGTGCAGCTCTCTGC 1740
 -----+-----+-----+-----+-----+
 ACGAATACTTTTAGTGCTGAATCCTTTTGTAGTTCTGAAAAGTAAACACGTCGAGAGACG

SfcI
 NlaIV |
 AceIII BsgI | AcI | CjeI
 BbvI RsaI | SfcI MspAI | TaaI |
 || | || | || |
 1741 TCTGGGTACTGCAACAACACTACAGATGTTCCAGCGGTTCCTACAGTAGCAACTCCTACGCA 1800
 -----+-----+-----+-----+-----+
 AGACCCATGACGTTGTTGATGTCTACAAGGTCGCCAAGGATGTCATCGTTGAGGATGCGT

CjeI
 RsaI |
 |
 1801 CTATGGGTATCAAGGTACTTGGGGAATGACTTGGGTTGATGATACGCAAGCACTCCAAA 1860
 -----+-----+-----+-----+-----+
 GATACCCATAGTTCCATGAACCCCTTACTGAACCCAACTACTATGGCGTTTCGTGAGGTTT

Bpu10I
 DdeI
 Hpy178III
 HinfI |
 TfiI |
 HgaI |
 Hpy188IX |
 BseMII |
 CviJI |
 CviJI |
 Tth111III
 DdeI
 MwoI |
 AluI |
 CviJI |
 Sau96I |
 AvaII |
 1861 GACTAAGACAGCGACATTAGCTTGGACCAATACAGGCTACCTTCCGAATCCTGAGCGTCA 1920
 -----+-----+-----+-----+-----+
 CTGATTCTGTCGCTGTAATCGAACCTGGTTATGTCCGATGGAAGGCTTAGGACTCGCAGT

AvaII
 EcoO109I
 Psp5II
 Sau96I
 Sse8647I
 A1oI
 CviJI
 Sau3AI
 DpnI
 BstYI |
 FokI | Hpy188IX
 AlwI |
 HinfI
 TfiI
 1921 AGGACCTTTAGTTTCTAATAGCCTTTGGGGATCTTTTTCAGACATCCAAGCGATTCAAGG 1980
 -----+-----+-----+-----+-----+
 TCCTGGAAATCAAGGATTATCGGAAACCCTAGAAAAAGTCTGTAGGTTTCGCTAAGTTCC

MwoI
 HinfI |
 BbvI |
 TaqI |
 DpnI |
 Sau3AI |
 Hpy188IX |
 AcI |
 Fnu4HI |
 CviJI |
 FauI |
 Sth132I |

PleI HinfI MnlI || || CviJI || TseI || ||
 TGT CATAGAGAGAAGT GCTTTTGACTCTTTGTTCAGATCGAGGCTTCTGGGCTGCGGGAGT
 1981 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2040
 ACAGTATCTCTCTTCACGAAACTGAGAAACAAGTCTAGCTCCGAAGACCCGACGCCCTCA

 PleI
 Tsp509I || DdeI TaaI
 CGCCAATTTCTTAGATAAAGATAAGAAAGGGGAAAAACGCAATACCGTCATAAATCTGG
 2041 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2100
 GCGGTTAAAGAATCTATTTCTATTCTTTCCCTTTTTTGCCTTTATGGCAGTATTTAGACC

 Hpy188IX BspI
 MnlI || Fnu4HI BspI || AluI
 HaeIV || CviRI || Hpy188IX || MseI || CviJI
 HinfI || TseI || HhaI BbvI || ||
 TGGATATGCTATCGGAGGTGCAGCGCAAACCTTGTCTGAAAACCTTAATTAGCTTTGCCTT
 2101 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2160
 ACCTATACGATAGCCTCCACGTCGCGTTTGAACAAGACTTTTGAATTAATCGAAACGGAA

 BstXI DdeI
 TTGCCAACTCTTTGGTAGCGATAAAGATTTCTTAGTCGCTAAAAATCATACTGATACCTA
 2161 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2220
 AACGGTTGAGAAACCATCGCTATTTCTAAAGAATCAGCGATTTTATAGTATGACTATGGAT

 CviJI NlaIV || BsmAI
 CviRI || BsbI MmeI DdeI ||
 TGCAGGAGCCTTCTATATCCAACACATTACAGAATGTAGTGGGTTTCATAGGTTGTCTCTT
 2221 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2280
 ACGTCTCGGAAGATATAGGTTGTGTAATGTCTTACATCACCCAAGTATCCAACAGAGAA

 CviJI
 ScrFI || HinfI PleI MnlI TseI || ||
 EcoRII || || Fnu4HI || ||
 AGATAAACTTCTCTGGCTCTTGGAGTCATAAACCCCTCGTTTTAGAGGGCAGCTCGCTTA
 2281 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2340
 TCTATTTGAAGGACCGAGAACCTCAGTATTTGGGGAGCAAAATCTTCCCGTCGAGCGAAT

 Bsu36I DdeI
 Hpy178III
 Eco57I ||
 BseMII ||
 MaeII MboII ||
 CviJI || Hpy188IX BstZ17I ||
 AceIII || DpnI || AccI ||
 BbvI || Sau3AI || BbsI || || MnlI || BciVI
 TAGCCACGTCAGTAATGATCTGAAGACAAAGTATACTGCGTATCCTGAGGTGAAAGGTTT
 2341 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2400
 ATCGGTGCAGTCATTACTAGACTTCTGTTTCATATGACGCATAGGACTCCACTTTCCAAG

 HphI MboII AluI
 MseI NlaIII || CviJI Hpy178III
 TTGGGGGAATAATGCTTTTAACATGATGTTGGGAGCTTCTTCTCATTCTTATCCTGAATA

```

2401 -----+-----+-----+-----+-----+ 2460
      AACCCCTTATTACGAAAATTGTACTACAACCCTCGAAGAAGAGTAAGAATAGGACTTAT

      BspMI                      Hpy188IX
      CviRI |                      HinfI |                      Hpy178III
      |      |                      TfiI |                      MaeII |
      |      |                      |      |                      |      |
2461 CCTGCATTGTTTGTATACCTATGCTCCATACATCAAAGTGAATCTGACCTATATACGTCA
      -----+-----+-----+-----+-----+ 2520
      GGACGTAACAAAATATGGATACGAGGTATGTAGTTTGACTTAGACTGGATATATGCAGT

      Hpy188IX                      DpnI
      AluI |                      BglII |
      CviJI |                      BstYI |                      MboII
      Hin4I |                      Sau3AI |                      MboII
      |      |                      |      |                      |      |
2521 GGACAGCTTCTCGGAGAAAGGTACAGAAGGAAGATCTTTTGATGACAGCAACCTCTTCAA
      -----+-----+-----+-----+-----+ 2580
      CCTGTGCAAGAGCCTCTTTCCATGTCTTCTCTAGAAAAGTACTGTCGTTGGAGAAGTT

      Hpy188IX
      MnlI |                      HinfI |                      BplI
      |      |                      HphI |                      |
      |      |                      |      |                      |
2581 TTTATCTTTGCCATATAGGGGTGAAGTTTGAGAAGTTCTCTGATTGTAATGACTTTTCTTA
      -----+-----+-----+-----+-----+ 2640
      AAATAGAAACGGATATCCCACTTCAAAGTCTTCAAGAGACTAACATTACTGAAAAGAAT

      BsrDI
      Hpy188IX                      DpnI
      DpnI |                      Sau3AI |                      SfcI
      Sau3AI |                      Hpy178III |                      CviRI
      |      |                      |      |                      |
2641 TGATCTGACTTTATCCTATGTTCTGATCTTATCCGCAATGATCCCAAATGCACACAGC
      -----+-----+-----+-----+-----+ 2700
      ACTAGACTGAAATAGGATACAAGGACTAGAAATAGGCGTTACTAGGGTTTACGTGATGTCG

      CviJI                      CviJI
      NlaIV |                      HaeI
      |      |                      HaeIII
      |      |                      StuI
      |      |                      MwoI
      |      |                      |
2701 ACTTGTAATCAGCGGAGCCTCTTGGGAAACTTATGCCAATAACTTAGCACGACAGGCCTT
      -----+-----+-----+-----+-----+ 2760
      TGAACATTAGTCGCCTCGGAGAACCCTTTGAATACGGTTATTGAATCGTGCTGTCCGGAA

      CviJI                      BsrI
      HaeI                      CviJI
      HaeIII                     HaeIII
      StuI                      EaeI
      MwoI                      GdiII
      |      |                     BsiHKAI
      |      |                     Bsp1286I
      |      |                     |
2761 GCAAGTGCGTGACGGCAGTCACTACGCCTTCTCTCCTATGTTTGAAGTGCTCGGCCAGTT
      -----+-----+-----+-----+-----+ 2820
      CGTTCACGCACGTCCGTCAGTGATGCGGAAGAGAGGATACAACTTCACGAGCCGGTCAA

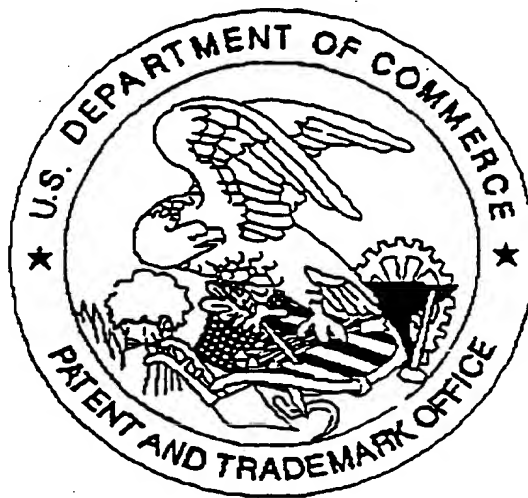
      DpnI
      NlaIV
      BamHI |
      BstYI |                      DpnI
      |      |                      BglII |

```

50110433.130153

45

United States Patent & Trademark Office
Office of Initial Patent Examination – Scanning Division



Application deficiencies were found during scanning:

☐ Page(s) _____ of Declaration were not present
for scanning. (Document title)

☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

☐ Scanned copy is best available.